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Studies in the Principles of Phytotoxicity

I. THE ASSESSMENT OF RELATIVE TOXICITY

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(*Received 12 September 1951*)

SUMMARY

The biological effects of phytotoxic compounds will be dependent upon uptake, the amounts reaching the site of toxic action, and the toxicity at cell level. When the materials are applied as sprays to growing plants absorption through the roots, retention by and penetration into the shoot, transport and localized accumulation are factors which may determine differences in response either between compounds or between species. The precise assessment of relative toxicity must therefore involve studies of the effects on whole plants and at cell level.

For such assessments it is essential to determine in the first place the change in the biological effects brought about by a wide range of dosage. The percentage inhibition of germination bears a sigmoid relationship to the amount of toxicant in the external medium, while the same relationship holds between percentage mortality and the concentration of the spray solution. Accurate comparisons of relative toxicity can only be obtained when the variation in response is measured at several dosages and the data treated by the methods of probit analysis.

Using such techniques in germination or spraying experiments it has been demonstrated that the relative toxicities of chlorinated phenoxyacetic acids, alkyl phenylcarbamates, dinitro-alkylphenols, pentachlorophenol, thioacetic acid, and formamide are greatly dependent on the species. In fact, the order of toxicity may be reversed from one species to another, while between compounds the results of germination tests may or may not be comparable with those obtained by spray applications.

The physical characteristics of the spray solution will in part determine the degrees of retention and penetration. For compounds with a low solubility in water the addition of a hygroscopic substance may increase the percentage kill. According to the species, spray solutions of a low surface activity may be more or less toxic than those with a high activity, while the relative effects of oil emulsions and aqueous sprays vary between species.

For compounds which are freely translocated, the methods of growth analysis are of value for assessing the toxic effects, especially of non-lethal dosages. Since the effects on the growth of the component parts of the plant may be widely divergent, conclusions based on a single criterion are likely to be erroneous. Where movement of the compound is restricted, such as with hydrocarbons, an assessment of toxicity can be obtained by measuring the degree of localized damage following on the application to the leaves of individual droplets of varying size.

Lemna minor has the twin advantages that the experimental conditions can be controlled and that in some respects its response to phytotoxic compounds is akin to that of unicellular organisms. Since with some compounds at any one dose the depression in the growth rate remains constant with time (e.g. nitrophenols),

while for others the depressant effect is cumulative (e.g. dichloro-phenoxyacetic acid, cupric salts), the nature of the growth response must first be established before comparisons between compounds can be made.

For studies of relative toxicity at cell level two methods have been employed. The external concentrations in the agar medium required to halve the growth rate of *Trichoderma viride* have been determined, or the dosages required to bring about a standard effect on the respiration of yeast have been estimated.

It is concluded that only by using a range of species and a number of techniques can relative toxicity be established with precision.

INTRODUCTION

THERE is no need to emphasize the rapidity of the advances that have been made during the last decade in developing techniques for the selective control of weeds. In the present phase many of the investigations are concerned with field trials to determine the most effective or most economic methods of application, much research relates to the empirical testing in the greenhouse and laboratory of new compounds for their possible toxic properties, and further research, but on a much smaller scale, aims at studying the principles which underlie selective toxicity and the nature of the toxic action.

A conspectus of the many hundred papers that have been published since 1946 can be obtained from the reviews by Norman, Minarik, and Weintraub (1950), and Blackman, Templeman, and Halliday (1951). From this extensive material it is possible to gain some idea of the broad differences in the toxic effects produced by different groups of compounds and to obtain some inkling of the physiological factors involved in selective action. It has been established that the over-all toxic effects, such as death, resulting from a standard experimental treatment may greatly alter with the stage of development of the plant. It is equally clear that the relationship between stage of development and the toxic effects will show different trends with different species, while such trends will also vary with different toxicants. Moreover, even within a group of closely allied compounds the order of relative toxicity may change not only with the species but with the stage of development of a single species (Blackman, Holly, and Roberts, 1949; Blackman, 1950 *a*).

For major groups, such as the substituted phenoxyacetic acids or the alkyl nitrophenols, some knowledge of the general relationships between toxicity and chemical structure has been acquired. Within these groups a variety of physiological and metabolic effects have been found, but in no instance has it yet been established what is the precise nature of the toxic action at cell level, or what are the major factors which operate in determining the differences between susceptible and resistant species.

On the basis of present findings it would appear that a successful assessment of the phytotoxic effects which result in death will, in many instances, be dependent on the integration of relatively small differences, and that for the measurement of such differences precise experimental techniques are demanded. From an examination of the work that has been published in the last seven years it is clear that much more information could often have been

obtained if greater consideration had been given both to the design and analysis of field experiments and to the techniques employed for measuring relative toxicity. Despite the fact that special techniques and special methods of statistical analysis have been devised for determining the comparative toxicity of insecticides and fungicides, there has been no general attempt to use such methods in studies of phytotoxicity.

The application of statistical methods is admittedly not an end in itself and it can only serve as a basis for the interpretation of results from experiments designed either to detect minor rather than major differences or to investigate the interactions between the factors which may be operating. The proper planning of experiments in turn will be greatly aided if the nature of the probable effects can be predicted. In two previous papers (Blackman, 1950 *a* and *b*) an attempt has been made to define the possible factors which may be involved in bringing about selective action when the toxicant is either added to the soil or applied as a dust or a spray to the shoots of a mixed population. Briefly it has been put forward that differences in susceptibility between species may be dependent on variations in (i) absorption through the roots, (ii) penetration into the shoot, (iii) the extent and rate of movement within the plant, (iv) the degree of localized accumulation in different tissues, and (v) the toxicity at cell level. In addition, when spray or dust applications are made there is the factor of the quantity retained by the shoot. This retention will be dependent in part on the physical characteristics of the applied material and in part on the size and shape of the leaves, the nature of the shoot surfaces, and the habit of growth.

It is on the basis of this analysis that research at Oxford has been in progress on the several aspects of the general problem relating to the assessment of relative toxicity. In each type of investigation methods are required which will allow of the precise measurement of the variables under consideration, and such precision cannot be achieved unless the nature of the response to the experimental treatment has first been established. Once the relative changes involved in each type of experiment have been determined, it should be possible to link the observations together. The general plan, therefore, has been to study on the one hand the effects on unicellular organisms or isolated pieces of tissue, and on the other the effects on whole plants with the object of resolving the over-all toxic effects into the individual parts of absorption, retention, penetration, translocation, accumulation, and toxicity at the site of action.

The present introductory paper is concerned with some of the methods that are being employed for the measurement and interpretation of the effects produced by different groups of phytotoxic substances.

EXPERIMENTAL RESULTS

The assessment of quantal effects. In order to evolve techniques for the precise evaluation of the toxic effect under consideration, it is first essential to determine the relationship between the response and dosage. Both on *a priori* grounds and on the basis of insecticidal and fungicidal research it would be

expected that the relationship between percentage mortality and the amount of toxicant will be sigmoid. For many types of phytotoxic substances it has been found that in terms of plant kill or the inhibition of germination a standard increment in the concentration produces a variable change in percentage mortality ranging from minimum responses at low and high concentrations and a maximum response at concentrations causing the death of half the population. It therefore follows that for the accurate comparison of relative toxicity the percentage kills produced by a single concentration of different compounds will be inaccurate, since such direct comparisons will only be valid if the mortality is linearly related to concentration. Many previous workers have overlooked this sigmoid relationship, especially in field tests of comparative performance, and have selected concentrations likely to give a high degree of kill: a selection which ensures high errors and a low validity for the conclusions which are reached.

On general considerations, it can be concluded that if by a suitable mathematical transformation percentage mortality could be linked with concentration by a linear regression, then the most precise measure of relative toxicity would be obtained from comparisons of the regression lines of the respective compounds. This statistical problem was first considered by Bliss (1937) for data relating the mortality of either insect eggs or insects to the dosage of individual insecticides. More recently, a comprehensive analysis has been published by Finney (1947).

It is not proposed to discuss the application of such techniques to the results of investigations on phytotoxicity since these will be treated fully in the following paper in this series. Briefly, it has been established that if the figures for percentage mortality are transposed to a scale of 'probits', then the converted values will be linearly related to the logarithm of the dosage. On the basis of these limits it is evident that for the most accurate comparison of two or more compounds the test plant should be treated with several concentrations of each compound, and the most accurate data will be obtained if the lowest concentration kills some plants and the highest does not kill all the plants. Moreover, in order to simplify the calculations the concentrations should increase on a geometric scale, so that when they are converted to a logarithmic scale the interval is equal. In practice it is best to carry out a preliminary trial to determine the concentration which kills approximately half the plants and subsequently so to order the concentrations that they are symmetrically distributed about this concentration. The emphasis on a 50 per cent. mortality—probit value 5·0—is based on statistical grounds since the dosage required to give this degree of mortality, as calculated from the regression and conventionally known as L.D. 50, produces the most precise estimate from the data.

The inhibition of germination. Experiments to determine whether a substance will inhibit or partially inhibit the germination of some selected species have been widely employed as a means of estimating relative toxicity, particularly the toxicity of synthetic growth regulators—see reviews by Norman

et al. (1950) and Blackman *et al.* (1951). In many of these screening or sorting tests only a single concentration of each compound has been used and accurate assessment of the differences has not been attempted.

In general, the seeds have either been germinated on filter-paper in contact with an aqueous solution of the compound or the seeds have been placed in

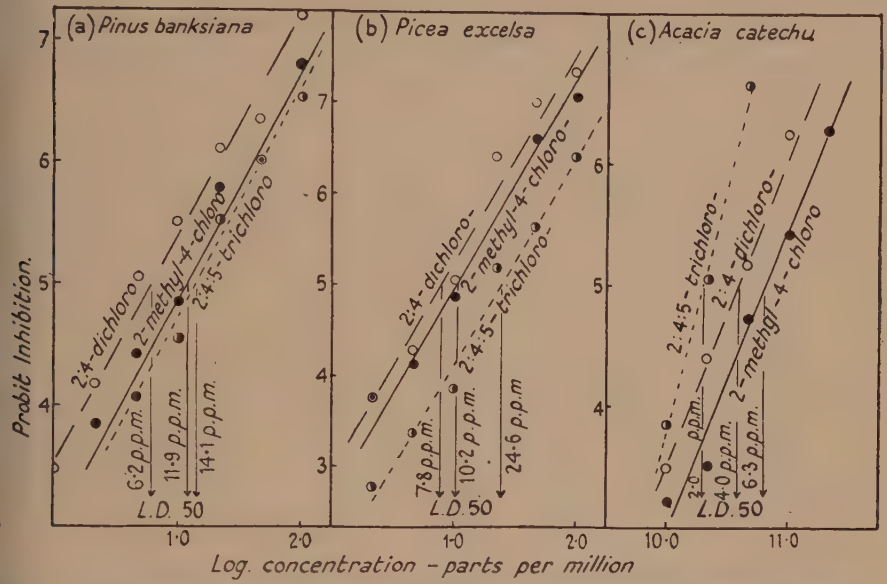


FIG. 1. The relative toxicity of sodium 2:4-dichlorophenoxyacetate, sodium 2-methyl-4-chlorophenoxyacetate, and 2 : 4 : 5 trichlorophenoxyacetic acid as measured by their power to inhibit the germination of (*a*) *Pinus banksiana*, (*b*) *Picea excelsa*, and (*c*) *Acacia catechu*. Percentage inhibition data transferred to probit scale where 5·0 equals 50% inhibition.

treated soil. For such tests in this laboratory, sand previously sterilized by heat to prevent mould infection has been selected as a standard medium. Where the compounds are readily soluble in water, the test solution is added to the sand in a Petri dish. In the case of less soluble compounds the required amount is taken up in a volatile organic solvent, such as acetone, and after incorporating this mixture with the sand the solvent is driven off by placing the sand in a warm oven. The standard quantity of treated sand is then placed with the seeds in Petri dishes, the water added, and the dishes transferred to an incubator.

Using these techniques, the effects of varying concentrations of substituted phenoxyacetic acids on the germination of a range of tree species has been investigated.[1] Selected results for *Pinus banksiana*, *Picea excelsa*, and *Acacia catechu*, converted to the probit transformation, are given in Fig. 1. It is evident from the regression lines that the relative toxicity of the three compounds is dependent on the species. For *A. catechu* (Fig. 1*c*) trichlorophenoxyacetic acid is the most toxic, followed in order by the sodium salts of

[1] Data from thesis for Diploma in Forestry by T. N. Srivastava.

dichloro and methylchlorophenoxyacetic acids. With *P. banksiana* (Fig. 1*a*) the dichloro substitution is more active than the other two compounds which have approximately equal effects. On the other hand, in the case of *P. excelsa* (Fig. 1*b*) the trichloro compound is the least toxic, but the remaining two compounds are of the same activity. Thus, on the basis of the concentrations required to produce a 50 per cent. inhibition of germination, and examining the results for one species at a time, conclusions could be reached that trichlorophenoxyacetic acid is relative to sodium methylchlorophenoxy— 3·1 times more toxic, 2·4 times less toxic, or approximately equally toxic.

Several workers have pointed out that the inhibition of germination is seldom complete and that in consequence some criterion of germination must be adopted to distinguish between germinated and ungerminated seeds. Usually a standard length of either the whole seedling or of the radicle has been selected, and for the relatively large seeds of these tree species a total length of 2·5 cm. was chosen.

With smaller seeds, a somewhat simpler procedure has proved satisfactory for some species, namely, the seeds are placed on the bottom of the Petri dish, covered carefully with the standard weight of sand, and the number of shoots emerging through the surface counted. Barnsley, using this technique, has investigated in this laboratory the effects of some dinitro-alkylphenols and alkyl-phenylcarbamates in inhibiting the germination of *Linum usitatissimum*, *Brassica alba*, *Medicago sativa*, *Sorghum vulgare*, *Avena sativa*, and *A. fatua*, and in order to ensure rapid germination of the last species the seed coat was removed prior to treatment.

The comparative effects of ethyl- and isopropyl-phenylcarbamates have been assessed by using a range of concentrations, applied initially to the sand in acetone and recording the subsequent percentage emergence. From Table I it is evident first that the quantities required to bring about a 50 per cent. inhibition differ widely between species. Secondly, it is apparent that the relative potency of the two compounds greatly depends on the species. For *L. usitatissimum* the isopropyl compound is over 30 times more toxic, while for *M. sativa* the factor is only 1·4.

TABLE I

The relative effects of ethyl- and isopropyl-phenylcarbamates in inhibiting the germination of seeds of different species

Species	Concentration (p.p.m.) for 50% inhibition		Relative toxicity $NHCOOC_3H_7$/ $NHCOOC_2H_5$
	$C_6H_5NHCOOC_2H_5$	$C_6H_5NHCOOC_3H_7$	
Medicago sativa	210	150	1·4
Brassica alba	130	53	2·5
Sorghum vulgare	390	120	3·2
Avena fatua	110	12	9·2
Avena sativa	54	3	18·0
Linum usitatissimum	125	4	31·3

To a lesser extent, a similar but less startling variation with species was recorded in the comparison of the ammonium salts of 3:5-dinitro-*ortho*-cresol and 2:4-dinitro-6-*secondary*-butylphenol, applied in this case as aqueous solutions or suspensions to the sand. The data of Table II demonstrate the range of concentration required to bring about a 50 per cent. suppression of germination. Sorghum and oats are least affected by both compounds, while *B. alba* and *L. usitatissimum* are the most sensitive. It is to be noted that while for four of the species the secondary butyl compound is more toxic, there is an indication for lucerne that this trend is reversed.

TABLE II

The relative effects of the ammonium salts of 3:5-dinitro-o-cresol and 2:4-dinitro-6-sec.-butylphenol in inhibiting the germination of seeds of different species

Species	Concentration (p.p.m.) for 50% inhibition: Methyl compound	Secondary butyl compound	Relative toxicity
Medicago sativa	44	48	0·9
Brassica alba	21	12	1·8
Linum usitatissimum	16	4	4·0
Avena sativa	513	207	2·5
Sorghum vulgare	435	80	5·4

This complex relationship between relative toxicity and species has also been found in a study of the differential reaction of mustard (*B. alba*) and *Beta vulgaris* to a wide range of compounds.[1] Part of the investigation was concerned with the effects on germination and the results for four of the compounds tested are shown in Fig. 2. It is evident that thioacetic acid and sodium pentachlorophenate are more toxic to mustard than to sugar-beet, that sodium chloride has an equal effect on both species, and that formamide is more toxic to *B. vulgaris*. It follows, therefore, that conclusions as to the relative potency of the four compounds will again be dependent on the species chosen.

Relative mortality from spray applications. By employing in germination tests a comprehensive series of crop plants and weed species it is possible to assess the potential value of a compound for bringing about a differential suppression of weeds when it is either incorporated in the seedbed or applied to the surface prior to the emergence of the crop. From tests of this type it cannot be concluded what will be the effects if the material is applied as a spray or dust in the post-emergence phase, for not only is susceptibility linked with the stage of development, but the compound may now enter both through the shoot as well as through the roots. To obtain such information actual spraying or dusting tests must be carried out.

[1] Data from unpublished thesis by F. E. Alder for th Degree of Bachelor of Science.

In the field variables, such as climatic changes, cannot be controlled and therefore in the analysis of the factors involved a greater precision can in general be attained by conducting such experiments in the greenhouse, using the pot-culture technique for raising the plants. In order to achieve reproducible results during any series of tests uniformity of plant material and the

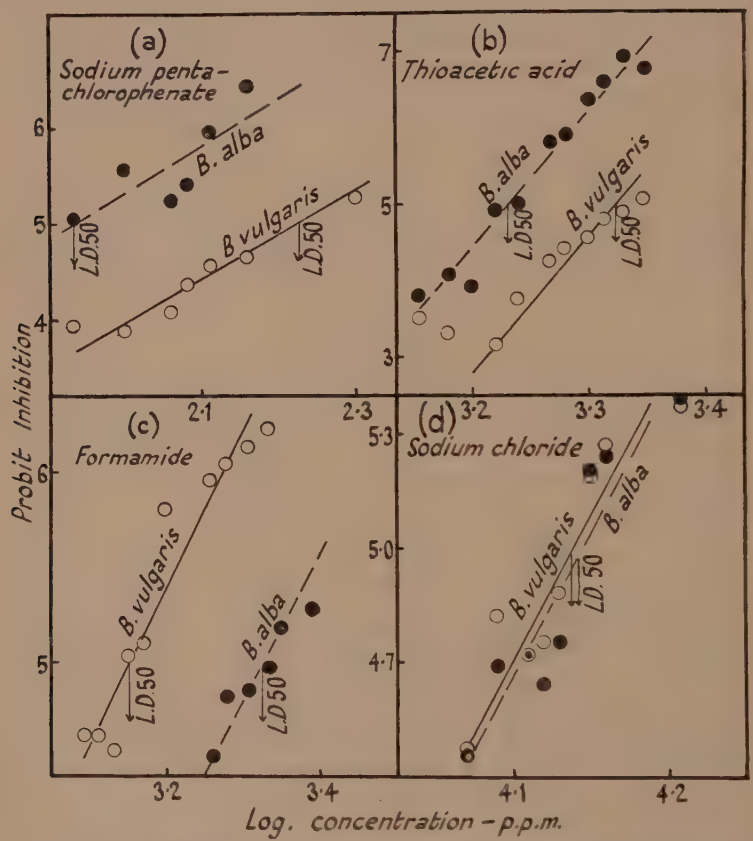
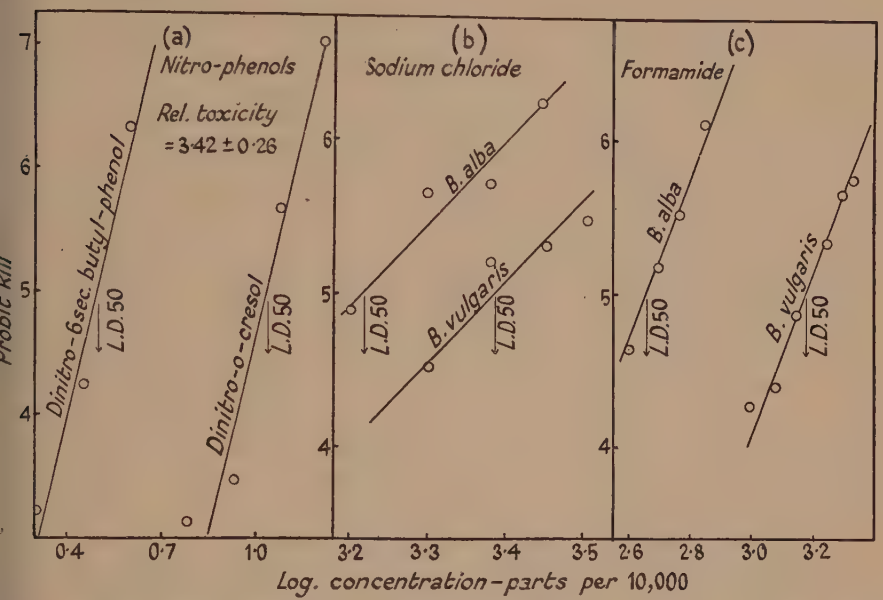


FIG. 2. The relative effects of (*a*) sodium pentachlorophenate, (*b*) thioacetic acid, (*c*) formamide, and (*d*) sodium chloride in inhibiting the germination of *Brassica alba* and *Beta vulgaris*. Percentage inhibition data transferred to probit scale.

means of application are essential. At Oxford the test plants are grown in a standard soil-sand medium in pots of aluminium and spraying is carried out when the plants have reached some selected size or sizes. Since the spray pattern and mean droplet size is dependent on the nozzle design and operating pressure, the same type of nozzle with a controlled and *constant* pressure has been used throughout. Alterations in the volume of spray reaching the pots has been obtained by varying the size of the nozzle orifice and the speed at which the nozzle moves across the pots.

Ideally, both before and after spraying the plants should be kept under uniform conditions of light intensity, temperature, and humidity. Since such

acilities are not available, and as greenhouse space is at present limited, the ots are normally placed in the greenhouse overnight and after spraying kept here a further 24 hours before being transferred to the open. With these mitations for critical comparisons all the spraying must be carried out in a ay and this has set a maximum of 200 pots for an individual experiment.



IG. 3. (*a*). The relative toxicity of 3 : 5-dinitro-*o*-cresol and 2 : 4-dinitro-6-*sec.*-butylphenol s measured by their power to kill young plants of *Linum usitatissimum*. (*b*) and (*c*). Specific ifferences in the amounts of either sodium chloride or formamide required to produce quivalent mortalities in seedlings of *Brassica alba* or *Beta vulgaris*. Percentage mortality ata transferred to probit scale. All compounds applied as aqueous sprays at a volume rate equivalent to 100 gallons per acre.

Where the aim is to compare the relative toxicities of a wide range of com- ounds, the simplest basis for comparison is the variation in the amount equired to bring about the death of half the plants, and these dosages can est be determined by using a range of concentrations and constructing a robit diagram.

The results of three experiments of this type, given in Fig. 3, illustrate how he conclusions may be the same or different from those based on the evidence f germination tests even when the species and compounds are common to oth sets of experiments. Inspection of Fig. 3*a* shows that to *L. usitatissimum* n the early vegetative phase dinitro-*sec.*-butylphenol in terms of plant ill is 3·4 times as toxic as dinitro-*ortho*-cresol, while a ratio of the same rder (*vide* Table II) has been arrived at from the results of the germination est. On the other hand, whereas sodium chloride causes an equal effect in hibiting the germination of *B. alba* and *B. vulgaris*, it is more toxic to mustard lants (Fig. 3*b*). An even greater discrepancy arises in the case of formamide:

in the germination phase *B. vulgaris* is more susceptible than *B. alba*, but when formamide is applied as a spray to young plants the relative susceptibility of the two species is reversed (compare Fig. 2*c* and Fig. 3*c*).

From the contrasting results of these germination and spraying tests it must be concluded that with the change of experimental conditions there is a reorientation of the factors which contribute to the relative toxic effects of sodium chloride and formamide. In the germination experiments the seeds are surrounded with a solution of the toxic compound and inhibition will be dependent on the quantity of toxicant adsorbed together with its intrinsic toxicity at cell level. Similarly, in the spraying trials, death will be dependent on the amount of compound reaching the sites of toxic action, and this in turn will be dependent upon a combination of the quantity of spray solution retained by the shoot and the mechanism and rate of penetration. Thus the disparity between the germination and spraying tests with sodium chloride could be explained on the basis that for both mustard and *B. vulgaris* in the germination phase the integrated effect of absorption and toxicity at cell level are the same for both species, while under the conditions of spraying less material enters the shoot of *B. vulgaris* due either to a smaller retention of the spray by the shoot or to a slower rate of entry—or a combination of both. Similar postulates can be put forward to explain the results for formamide if it is assumed that under both sets of conditions there are specific differences both in absorption and toxicity at cell level.

The degree to which retention and penetration operate in determining differences between species can be most readily investigated where the toxicant has the same effect at cell level, irrespective of species. Such conditions are most likely to be approached with sulphuric acid since at the high concentrations which will differentially kill many dicotyledonous weeds in cereal and onion crops, destruction of the cytoplasm will inevitably occur in those cells which come into direct contact with such highly acidic solutions. In consequence, selective action in the early vegetative phase will be dependent on the degree of cell destruction, especially the killing of the primary meristems. Differences between species will therefore be dependent on the morphology of the shoot, the nature of the epidermis, and whether there is an exposed apical bud or a basal protected meristem (Åslander, 1927; Blackman and Templeman, 1936; and Blackman *et al.*, 1949).

Retention and penetration of the spray will not only be related to specific differences in morphology but also to the physical characteristics of the droplets. The divergent effects of the addition of a surface active agent on the kill of different species brought about by solutions of sulphuric acid are shown in the upper part of Table III, where the figures given are the means of four experiments. In the case of *Chenopodium album*, with its peltate hairs on the epidermis, the increase in mortality caused by a reduction in the surface tension can be ascribed to a combination of greater retention and increased penetration. The contrasting decrease in the kill of *Capsella bursa-pastoris* following on a decrease in the tension at the lower concentration must be

ue to any increase in penetration being more than offset by a lower retention, g. if a continuous film takes the place of discrete droplets, the solution will ore readily run off the shoot. For *Senecio vulgaris* two alternative explanaons can be given: either variations in penetration or retention are not closely nked with changes in the surface activity or a positive effect on penetration balanced by a negative effect on retention.

TABLE III

he interrelationship between species and the surface activity of the spray solution in determining degree of mortality

Species	Concentration of sulphuric acid			
	9·2%	9·2% and wetting agent	13·8%	13·8% and wetting agent
henopodium album . .	66·0	95·0	83·0	100·0
apsella bursa-pastoris . .	82·7	67·6	88·6	88·6
enecio vulgaris . .	88·7	89·2	91·3	92·3

	Concentration of sodium chloride			
	12·0%	12·0% and wetting agent	16·0%	16·0% and wetting agent
rassica alba . . .	48·1	81·6	66·5	89·6
eta vulgaris . . .	16·5	4·3	24·7	21·0

(Spray solutions applied at a rate of 100 gallons per acre.)

Similar specific differences have also been obtained when a surface active gent is added to sodium chloride (see lower part of Table III). With *B. lba* a decrease in the surface tension caused a greater spread of the droplets ver the leaves and brought about a greater mortality, while the spray tended run off the smoother leaves of *B. vulgaris* and the percentage kill was ecreased.

With compounds of a low vapour pressure the available evidence suggests at in aqueous sprays entry is confined to the epidermis, and that penetration largely arrested once the spray droplets have dried out and the substance is eposited on the leaf surfaces (e.g. Rice, 1948). It follows, therefore, that the mount entering the leaf will be related to the mean area of contact and the me taken before deposition takes place, and the length of this period in turn ill be related to the humidity and rate of movement of the surrounding air. hus surface active agents by increasing the area of contact will accelerate enetration, but on the other hand the replacement of discrete droplets by a in film may also have a reverse effect since the time elapsing before deposion takes place will be diminished. Again, substances or derivatives which are aringly soluble in water will be less active than compounds of a high soluility, especially if the conditions favour a high rate of evaporation. In conseuence, it would be expected that the addition of some non-toxic hygroscopic

substance would bring about a greater penetration, more particularly fo compounds of a low solubility in water.

On the basis of these considerations a further examination has been made c the effects of inorganic chlorides on *B. alba* and *B. vulgaris*. Since magnesium chloride is considerably more soluble in water than either sodium or potassium

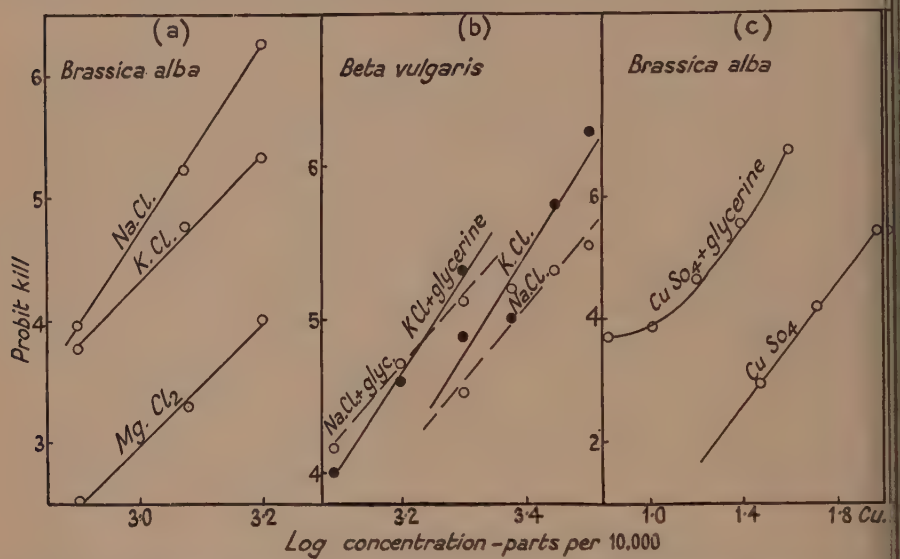


FIG. 4. (*a*). The relative toxicities of sodium, potassium, and magnesium chlorides when applied as aqueous sprays containing 5·0 % glycerol. (*b*) and (*c*). The increase in toxicity caused by adding 5·0 % glycerol to spray solutions containing either potassium and sodium chlorides or cupric sulphate. Percentage mortality data transferred to probit scale. Spray applications made at a volume rate equivalent to 100 gallons per acre.

chloride, it could be advanced that if these chlorides all acted in the same way then the magnesium salt would be the most toxic. It would also be expected that these differences would largely disappear if a hygroscopic substance was added to the spray solutions. In point of fact the experimental results are completely at variance with such hypotheses since with both species magnesium chloride is the least toxic. For example, while in one experiment sodium and potassium chloride at a concentration of 32 per cent. killed 68·2 and 88·6 per cent. of bean seedlings, the mortality for magnesium chloride was 48·4 per cent. Again, it is evident from Fig. 4*a* that when glycerol is added at a concentration (5 per cent.) which by itself is not injurious, then in their toxicity to *B. alba* there are still marked differences between magnesium and the other two chlorides.

From these results the conclusion is reached that there is no common effect of these chlorides. From Fig. 4*b* it is evident that for both sodium and potassium chloride the physical characteristics of solubility may limit the over-all toxic effects since the addition of glycerol has brought about an increased toxicity to *B. vulgaris*.

It should also be noted that the probit regression lines for the combined

lt and glycerol treatments are parallel to those for the chlorides alone and us it can be inferred that the mode of action is not affected by the addition of ycerine (see Finney, 1947). The data of Fig. 4*c*, which are taken from a study the comparative toxicity of cupric salts,[1] have been included to emphasize at this lack of interaction cannot always be assumed. While it is clear that e inclusion of glycerol has greatly increased the toxicity of cupric sulphate *B. alba*, the probit relationship of kill with the logarithm of dosage no nger holds for a mixture of cupric sulphate and glycerine.

So far consideration has been given to some of the factors which govern the tention and penetration of compounds applied to shoots as aqueous solu-ons, but there are a number of phytotoxic substances which are either soluble in or immiscible with water. Many such compounds can be incor-rated in mineral or vegetable oils, e.g. the esters of substituted phenoxyacetic ids, and they are often applied in the field as oil-water emulsions. From hat has already been stated it would be anticipated that the retention of an nulsion may be different from that of a water solution, since the differences the physical properties of the droplets and subsequently in the components hen the emulsion breaks will clearly affect the spread over the shoot and the ea of contact. Again, the rate of penetration will be dependent on the rate entry via the oil and water phases and the partition coefficient of solubility tween the two phases.

As an example of the large differences that may arise when an oil emulsion substituted for an aqueous spray, the results of a field experiment are given Fig. 5, in which dinitro-*o*-cresol and dinitro-*secondary*-butylphenol were plied either as suspensions in water or as emulsions containing 10 per cent. refined Arachis oil. Taking first the data for *Raphanus raphanistrum* it is ident that on the basis of the concentrations required to bring about a per cent. mortality the emulsions of both compounds are much more xic than the water suspensions. It is also apparent that the butylphenol mpound is more toxic than the cresol and it should be observed that while in ater the ratio of relative toxicity is 9·65, in the emulsion the ratio is lower, mely, 6·43. Lastly, it should be noted that the regressions for the oil nulsions have a steeper slope, thus indicating that the general mode of tion is different from that of water suspensions.

In many respects the results for *Chrysanthemum segetum* are widely diver-nt from those for *R. raphanistrum*. Instead of the greater toxicity of the tylphenol being decreased by a change from water to an oil emulsion as a rrier, it is accentuated, i.e. the ratio of relative toxicity between compounds anges from 4·06 to 7·21. Again, the regressions for the oil-emulsion treat-ents have smaller, not greater, slopes than those of the regressions for ueous treatments. Thus for this species and for *R. raphanistrum* it is not ssible to obtain a direct comparison of the toxicity of either compound in ater as against in an oil emulsion since the ratio will change with the level of rtality selected.

[1] Unpublished thesis by J. S. Sands for the Degree of Bachelor of Science.

These divergent trends again emphasize the interdependence of the characteristics of both the spray and of the species in determining the relative phytotoxic effects of compounds, even when they belong to the same series.

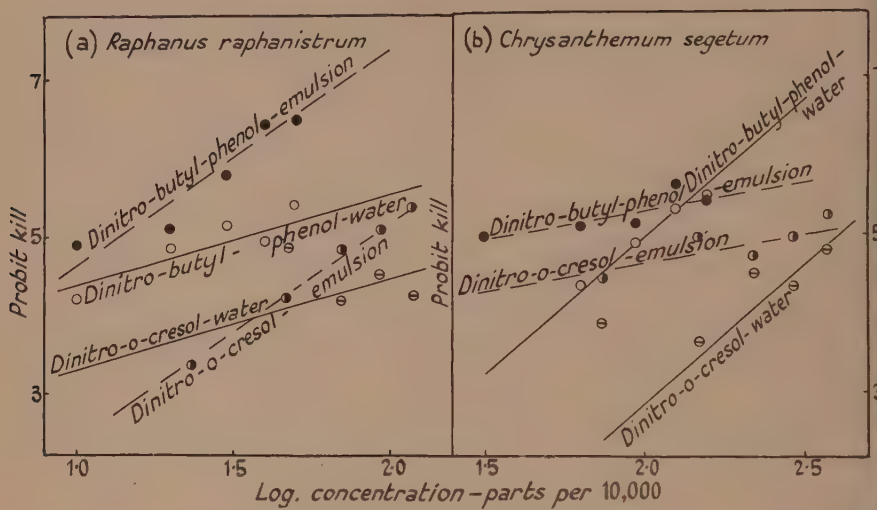


FIG. 5. The relative toxicities of 3:5-dinitro-*o*-cresol and 2:4-dinitro-6-*secondary*-butylphenol when applied in water or in emulsions containing 10% of Arachis oil. Percentage mortality data for (*a*) *Raphanus raphanistrum*, and (*b*) *Chrysanthemum segetum* transferred to probit scale. Spray applications made at a rate equivalent to 100 gallons per acre.

The assessment of qualitative effects. From the viewpoint of the final selection of compounds which already show promise as potential herbicides, investigations which seek to establish the conditions under which the phytotoxic effects result in the end-point death are often the most appropriate. The criterion of mortality is clear-cut and on such a basis comparisons can be made between compounds which may differ widely in their effects and in their modes of phytotoxic action. When, however, it comes to an analysis of the changes that take place before and during the process of dying, the observations or types of test which are likely to be the most informative will vary greatly between different compounds since between groups, if not between individual compounds, there will be divergent effects on growth and morphological development, on the internal, biochemical, and physiological systems, and on cell organization.

Norman *et al.* (1950) have enumerated and commented on a number of techniques which have been developed for assessing differences in toxicity in terms of the changes in growth and development. Most attention has been given to the assessment of the type of effect produced by synthetic growth regulators, such as dichlorophenoxyacetic acid or the alkylphenylcarbamates. Observations have been made on the epinastic effects, the inhibition or depression of growth of the shoot of the leaves or of the whole plant. The

ompounds have either been applied to the soil, to the shoot in the form of a pray, as individual droplets placed on the apical bud or leaves, and as smears r bands of a lanoline mixture. Comparisons between compounds have in ıost instances been restricted to a single dose level with its attendant errors. 'his limitation of dosage, however, has not been the case in the investigation f Brown and Weintraub (1950), who have sought to evaluate quantitatively ıe 'formative' activity of growth-regulator compounds by measuring the xtent to which leaf growth in the kidney bean is suppressed following on the pplication to the apical bud of varying amounts of each compound. Likewise Vort (1951) has examined the effects of four concentrations of 2:4-dichloro-henoxyacetic acid on the growth and composition of *Fagopyrum esculentum*.

Application of methods of growth analysis. In all these investigations no ttempt has been made to employ the methods of growth analysis originated y Gregory (1917), Blackman (1919), and Briggs, Kidd, and West (1920) in ıe elucidation of the effects induced by phytotoxic substances. The paper by \sana *et al.* (1950) is, however, an exception since these workers assessed the ffects of dichlorophenoxyacetic acid on the relative growth rate and net ssimilation rate (based not on leaf area but on leaf weight) of two varieties of /heat. On the other hand, the interval between sampling occasions was large –30 days or more—and the experimental design was restricted to a compari-on between control plants and those treated with a single dose.

In this laboratory R. C. R. Cuninghame and the author are using the tech-iques of growth analysis to study the development of young plants of *Ielianthus annuus*, subsequent to treatment with sodium dichlorophenoxya-etate applied either as an aqueous spray to the whole plant or as individual roplets to the leaves or apical bud. The results of one spraying experiment re given in Fig. 6 to illustrate how divergent may be the changes induced in ıe component parts and in the whole plant. In order to obtain the *mean* ffects over the experimental period the relative growth rates—percentage hange in dry weight per day—for the whole plant, for root and stem, and for eaf area and leaf weight were first calculated, and for comparative purposes ıese growth rates have been expressed as a percentage of the corresponding gure for the control plants. Similarly, the values for net assimilation rate xpressed as gain in dry weight of whole plant per square decimetre of leaf per eek were first derived and then converted to a percentage of the control alue.

Considering first the changes in the several parts of the plant, it is apparent ıat over all concentrations the degree of inhibition of growth has been greatest or the roots, none at all for the stem, and intermediate for leaf weight. Since ıese varying degrees of inhibition will be integrated in the inhibitory effect ı the growth of the whole plant it would be expected that this reduction should ccupy an intermediate position between the maximal effect on the roots and ıe minimal effect on the stem.

It is also evident that, next to the root, the largest depressions have occurred either total leaf area or the area of the two leaves below the apical bud, and it

would also seem that leaf area is more affected than leaf weight. Changes in the leaf area and changes in the growth of the component parts of the plant will contribute to the value of the net assimilation rate which diminishes with increasing dosage in much the same way as the relative growth rate of the whole plant.

Thus conclusions as to the effects of increasing concentration of this growth

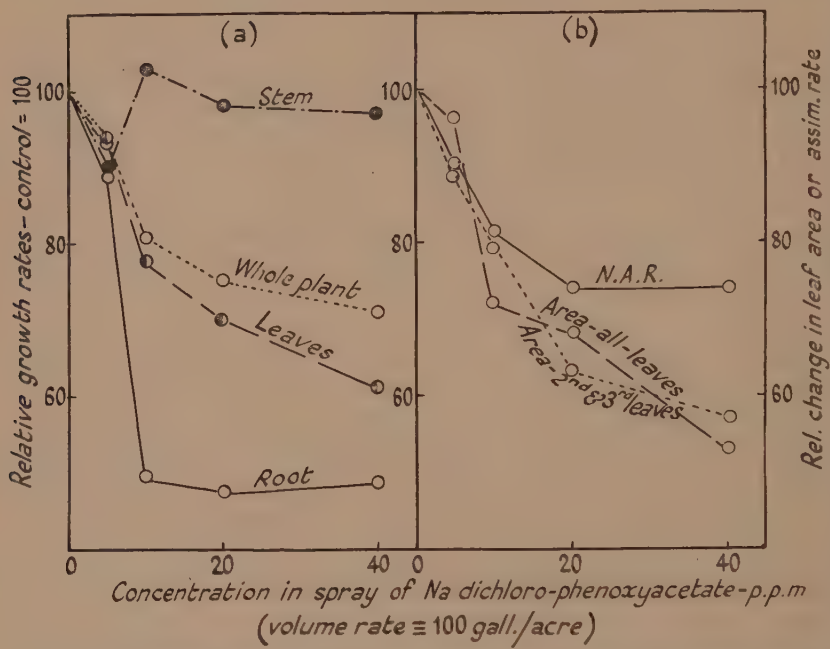


FIG. 6. The relative changes in (*a*) the growth rates of the component part of *Helianthus annuus*, and (*b*) the net assimilation rate and leaf area subsequent to spray applications of sodium 2:4-dichlorophenoxyacetate. Significant differences between treatments (P = 0·05); root 26·8, stem 12·8, leaf weight 11·7, whole plant 12·7, area (all leaves) 15·0, area (2nd and 3rd leaves) 16·5, N.A.R. 12·8.

regulator will differ widely according to the data selected for comparative purposes.

Application of individual droplets. An examination of the phytotoxic effects by means of the techniques of growth analysis will have most value for those groups of compounds which are freely distributed within the plant and where at sublethal dosages the toxic action is slow but progressive. There is, however, considerable evidence that for many other toxic compounds such as the alkyl-dinitrophenols or some hydrocarbons, transport within the plant is limited and active translocation is not generally involved. In consequence, injury tends to be localized in the cells adjacent to the points of entry through the leaf surfaces. It follows therefore that, if a droplet is placed on the leaf and the extent of the zone of injury can be estimated, then the resulting data will

provide a measure of the combined effects of the amount of penetration into the leaf and of the zone in which the effective concentration is such as to bring about appreciable injury.

By means of a micro-pipette, single drops can readily be applied to the surfaces of leaves and, if a large drop is demanded, too great a spread of

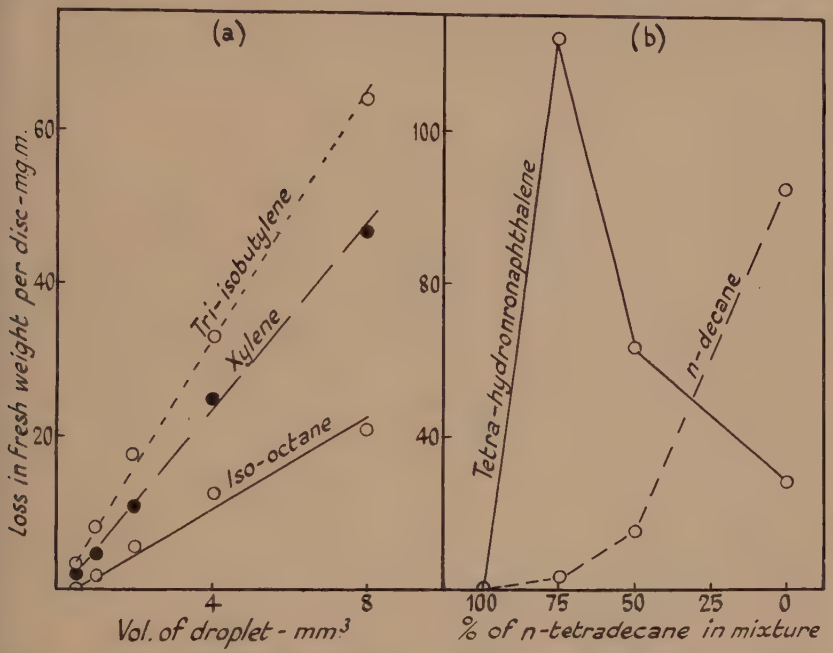


FIG. 7. (*a*). The relationship between the degree of injury and the volume of the droplet of hydrocarbon placed on the leaf. (*b*). The effects of adding *n*-tetradecane in differing amounts to a constant quantity (8 mm.) of either *n*-decane or tetrahydronaphthalene.

mobile liquids along the vein channels can be prevented by placing it in the centre of a small gelatine ring which has been pressed on to the leaf surface. When applications of dinitroalkylphenols are made in this way to the leaves of the pea or runner bean, a circular greyish-green zone develops and comparisons can be made by measuring the diameters.

With many of the hydrocarbons so far investigated the injurious effect is far more pronounced since in the affected area there is a breakdown of the chlorophyll. If this zone together with some of the visually unaffected and peripheral tissue is punched out with a cork borer and the weight of these samples is compared with similar sized areas from untreated leaves, then it has been found that over a considerable range of droplet size the difference in weight is nearly related to the external dosage.

The data of Fig. 7 have been selected from current investigations by G. W. Ivens which have as their aim an analysis of the factors which govern the selective action of mineral oil fractions when they are applied as herbicides to umbelliferous crops or forest nursery beds of coniferous species (Ivens,

Woodford, and Blackman, 1951). Fig. 7*a* shows clearly the linear relationshi between effect and dosage and the differences in toxicity to bean (*Phaseolu multiflorus*) leaves of three hydrocarbons. Fig. 7*b* illustrates the interaction which arise when one hydrocarbon is added to another. If tetrahydro naphthalene is mixed with *n*-tetradecane, which itself is non-toxic, then such mixtures cause a greater injury to the leaf tissue than tetrahydronaphthalen alone. On the other hand, when *n*-tetradecane is added to *n*-decane, instea of the toxicity being increased it is reduced. Thus it is evident that for a prope understanding of the toxicity of mineral oil fractions it is not sufficient to stud the individual effects of the possible constituents.

Lemna minor as a test organism. Although the droplet technique may giv an indication of cell injury subsequent to penetration, the observed differ ences between compounds will also include the differences in the amount penetrating through the leaf surface, which in turn will be related to th rapidity with which the droplets dry out, or if the compounds are volatile th rate at which they evaporate. Therefore, in any attempt to evaluate technique for the study of toxicity at cell level, variation in the external factors governin penetration should be reduced to a minimum. In addition, for a comparis of compounds which differ widely in the extent of their movement within th tissues, the test organism should be small.

Offord (1946) was the first to point out that *Lemna minor* might be used i 'screening' tests to select compounds with a potential value as herbicides, bu he did not investigate the conditions under which this species can be used fo an analysis of toxic action. *L. minor* has the advantage that it is a floweri plant with a high ratio of surface area to volume and little if any cuticle i present on the under surface which will be in contact with the nutrient solu tion containing the toxic agent. Moreover, as a result of the extensive investi gations of Ashby, Oxley, White, and Steinberg (e.g. Ashby and Oxley, 1935 Ashby *et al.*, 1949; White, 1937; Steinberg, 1941) the effects of light, tem perature, and nutrient supply on growth have been esablished with precision Furthermore, since reproduction is normally vegetative, homozygous materia can be obtained by starting with a single plant and building up and main taining a population for as long a period as the investigation demands.

The experimental procedures finally adopted will only be recorded briefl since full descriptions will be given in subsequent papers. A standardized bu small number of plants are placed in individual beakers containing a relativel large volume of nutrient solution to which the appropriate quantity o toxic material has been added. In order to prevent contamination and als to maintain both the pH and the external concentrations relatively constan the solutions are changed every 48 hours. The beakers are placed in water baths such that they are three-quarters immersed in water of which the tem perature is controlled and the beakers illuminated from above by a battery o fluorescent tubes.

The observations made on the growth of the plants have depended in pa on the nature of the investigation. Normally the number of fronds has bee

counted at frequent intervals; the change in dry weight has been determined by withdrawing beakers and drying the plants in partial vacuum at 55° C.

Before comparisons can be made between compounds it is essential to investigate the nature of the response with varying concentration, and such studies of different compounds have or are being made by several workers in

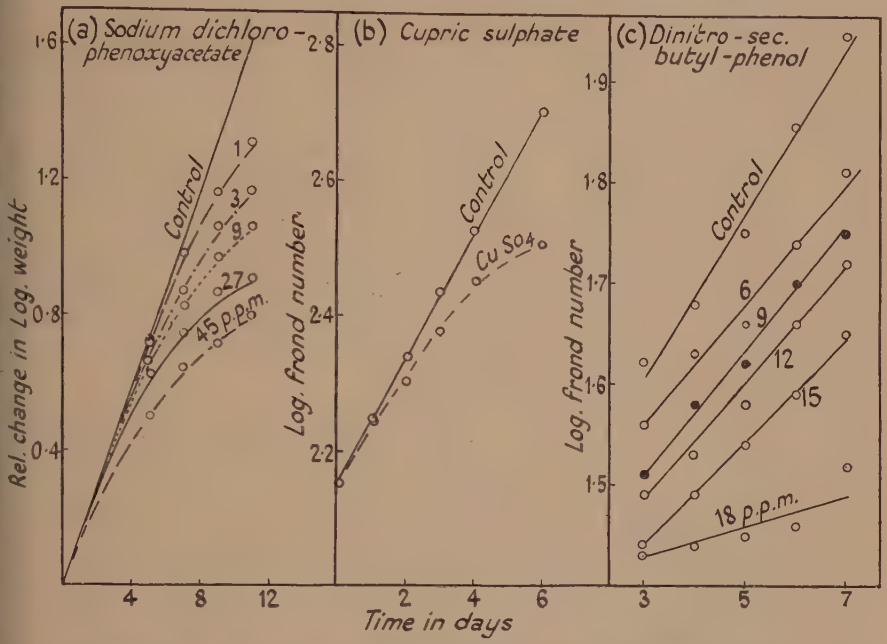


FIG. 8. Changes with time in the relative growth rate of *Lemna minor* following on treatment with (*a*) sodium 2:4-dichlorophenoxyacetate, (*b*) cupric sulphate, and (*c*) 2:4-dinitro-6-*sec*.-butyl phenol.

this laboratory. For example, the effect of sodium dichlorophenoxyacetate on the changes in dry weight are given in Fig. 8*a*. Since growth is exponential for the controls there is a linear relationship between the logarithm of weight and time, but for the various dosages of this growth regulator the relationships are not linear but curvilinear, thus indicating that the toxic effect is cumulative. In consequence, the relative reductions in growth brought about by the different concentrations will not be independent of time.

Because of the nature of this response, the selection of criteria to compare either differences between compounds or changes in the environmental conditions presents difficulties. Where the compounds or varying experimental conditions produce similar responses, the strictest analysis demands fitting a family of curvilinear equations. Alternatively, for the purposes of a sorting test, a less accurate but much simpler approach is to select a standard experimental period, determine the dry weight at the end of the period, and plot the difference in weight—on a logarithmic scale—between the initial and final sampling occasion against the logarithm of concentration. From the

freehand fitted curve the concentration required to bring about some standard reduction can be read off. As a modification of this technique, if the range of concentrations of a substituted phenoxyacetic acid is increased and the experimental period restricted to 2–3 days, then the relationship between percentage kill and concentration can be assessed where the criterion of death is the absence of chlorophyll and the inability of the plants to recover when transferred to an untreated nutrient solution.

With *L. minor* the synthetic growth regulators produce typical epinastic effects, frond size is reduced, and the plants tend to take on a hemispherical shape. With other compounds which do not bring about such changes it is possible to take advantage of the fact that during growth frond number is closely correlated with total weight, and counts of frond number have in part been used in an investigation of the relative toxicity of copper salts. In this study a somewhat modified procedure has been adopted in order to avoid chemical reaction between the salts and the components of the nutrient media. During each 48-hour period the control plants are transferred to distilled water for 4 hours, while the remainder are placed in distilled water containing the appropriate concentration of cupric salt.

Fig. 8*b* shows that while the rate of increase in frond number for the control plants is exponential, this is not so for plants placed in cupric sulphate (0·1 p.p.m. copper) for 4-hour periods every third day. In fact the curvilinear relationship is very similar to that found for sodium dichlorophenoxyacetate.

L. minor has also been employed in a study of the toxicity of dinitrophenol and with this group of compounds the effects on growth differ from those previously described. When the plants are placed in nutrient solution containing concentrations which depress growth, then after an initial phase of adjustment which extends for a period of a few days, growth subsequently continues exponentially but at slower rates than that of the controls—see Fig. 8*c*. It is therefore possible for the several concentrations and the control to calculate the linear regressions of growth with time and so determine the growth rates, i.e. the '*b*'s' of the regressions. The growth rates, expressed as percentages of the control, can then be plotted against the logarithm of the concentration and the concentration required to halve the growth rate determined. It has, however, been observed that at the higher concentrations in particular some of the plants die at irregular intervals while others continue to produce daughter fronds. In consequence, the mean growth rate is for a mixed population of live, dying, and dead plants. On these grounds, therefore, for the precise determination of small differences a standard of less than a 50 per cent. reduction has advantages, since under such conditions the errors due to the presence of moribund plants will be minimal.

A comparison of Fig. 8*c* with Fig. 8*a* emphasizes the point already made that it is essential before comparing compounds to establish the nature of the response. It is clear that a direct comparison cannot be attempted where in one instance the response is linear and in the other curvilinear.

Trichoderma viride as a test organism. Simon and Blackman (1949) have

mphasized that in investigations of the biological activities of weak organic cids the most comparable information can be obtained when the pH of the ıedium containing the compound under investigation is two units below that f the pK value. For studies, therefore, of substituted phenols organisms

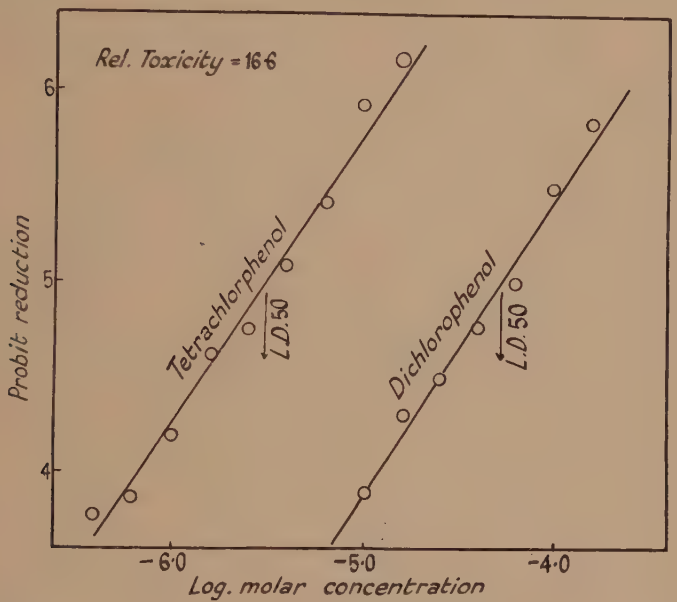


FIG. 9. Reductions in the growth rate of *Trichoderma viride* caused by varying concentrations in the agar medium (pH = 3·0) of 2:4-dichlorophenol and 2:4:5:6-tetrachlorophenol. Percentage reduction of radial spread transferred to probit scale.

hich will tolerate acidic conditions are desirable and for this purpose the ıould *Trichoderma viride* has been used since it will grow in an agar nutrient ıedia of pH 3. Moreover, *T. viride* is a simple organism with non-septate yphae and lends itself to an investigation of the relative toxicity at cell level.

In investigations of the differences in toxicity between chloro- and nitro-henols the standard technique of measuring radial growth of the mycellium ross an agar medium has been employed. The details of the method have een given previously (Simon and Blackman, 1949). Since for both nitro- and ıloro-phenols the rate of spread is linearly related to the concentration of xicant, the percentage reduction can be obtained by a comparison with the owth rate of the controls. These percentages can then be converted to obit values, and when these are plotted against the logarithm of the dosage the ints fall on a straight line.

As an example of the results obtained in this way data for 2:4-dichloro-ıenol and 2:3:4:6-tetra-chlorophenol are given in Fig. 9. It is evident at the relative toxicities required to halve the growth rate can be determined ith accuracy.

Yeast as a test organism. It has already been stressed that for the assessment of relative toxicity there is a considerable advantage in selecting species which have been already widely studied. As an example of a unicellular organism yeast therefore has these advantages, especially for those compounds

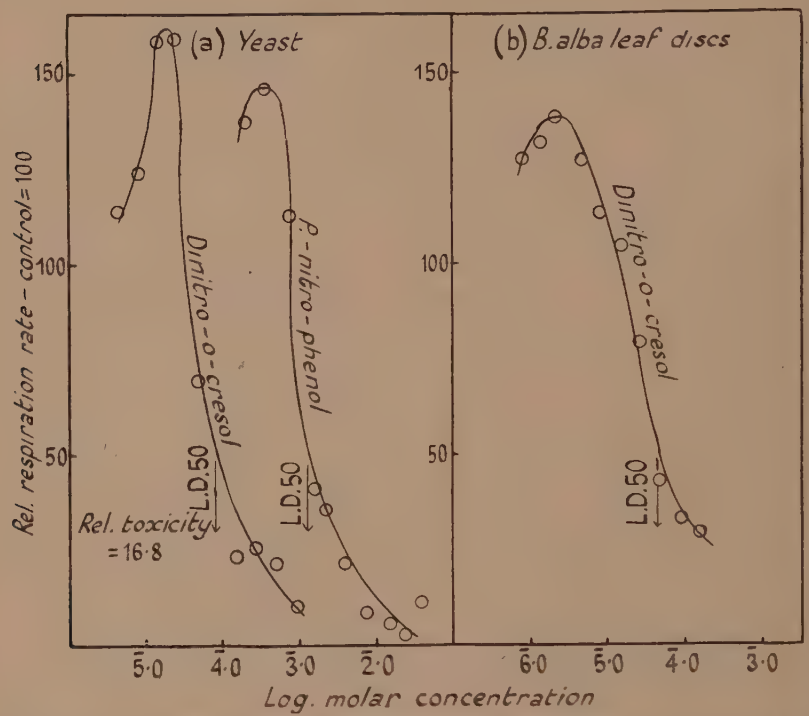


FIG. 10. The effects of varying concentrations of nitrophenols at pH 4·7–4·8 in altering the respiration rate (oxygen consumption) of (*a*) yeast, and (*b*) infiltrated leaf disks of *Brassica alba*.

such as the substituted phenols which are known to affect respiration. Using the Warburg technique the relative activity of a wide range of nitrophenols has been investigated, where the response has been measured by determining the concentration which halves the rate of oxygen consumption. Since at low concentrations the oxygen uptake is increased (Simon and Beevers, 1951) the change in the respiration with increasing concentration is best determined by fitting a freehand curve to the data and reading off the concentration required to reduce the respiration by 50 per cent. An example of the type of result obtained is seen in Fig. 10*a*.

Because the factors concerning the respiration of yeast have been so extensively investigated, further study may well provide an understanding of the nature of the toxic action of nitrophenols. It might be advanced that either for such an investigation or for the determination of relative toxicity, yeast is very different from the cell tissue of higher plants. On the other hand, it has

been stressed (Blackman *et al.*, 1951) that the effects on respiration are common to a wide range of plant and animal tissues. For example, when small disks of the leaves of *B. alba* are punched out with a cork borer and infiltrated under partial vacuum with solutions containing nitro-phenols, then the effects of increasing concentration on the respiration exhibit the same trend—compare Fig. 10*a* and *b*.

DISCUSSION

The present results serve to emphasize that for the precise assessment of the differences in toxicity between compounds, experiments cannot be confined either to an isolated technique or to a single species. It has been demonstrated within such widely divergent groups as the chlorophenoxyacetic acids, the alkylphenylcarbamates, and the dinitro-alkyl phenols that the relative toxicities expressed in terms of the power to inhibit germination are greatly dependent on the species (Fig. 1, Tables I and II). It has also been shown that even when the same organisms and the same compounds are compared the results for different tests give completely contrasting results, e.g. the relative toxicity of formamide to *B. alba* and *B. vulgaris* in the germination and spraying experiments (Figs. 2*c* and 3*c*).

Such a reversal in the trend, it has already been pointed out, may well be ascribed to the change in the experimental conditions between the two methods of assessment. From the analysis of some of the factors which will operate in spraying trials it is evident that both between species and between compounds the degree of both retention and penetration may play a major role in determining either the relative susceptibility of the species or the relative effects of compounds. Thus the external environmental conditions, the physical characteristics of the spray droplets, the nature of the leaf surfaces, and the morphology of the shoot must all be taken into account. It therefore follows that unless these variables are considered, errors or wrong conclusions will result.

The addition of a surface active agent may give different effects with different species (Table III). Alternatively, if a surface active agent is added to the spray solution as a matter of routine, and this has been done by some previous workers, differences in the surface activity of the compounds themselves may be masked. Again, if in the course of compounding products which lend themselves to extended agricultural usage, materials with hygroscopic properties are added, these may effect the toxicity of the active agent. Moreover, as the results for the addition of glycerine to sodium chloride or cupric sulphate (Fig. 4*b* and *c*) show, the addition of the same compound to different toxicants may bring about diverse reactions. Lastly, from Fig. 5 it is apparent how divergent may be the effects of aqueous suspensions and oil emulsions, and how much such differences will vary with the species. From previous investigations (Blackman, 1950*a*) it is known that the degree of retention of an emulsion may be different, while in Fig. 5 the variations in the slopes of the regressions suggest that the mode of action is not the same as that for the

water suspensions. On this evidence, considerable difficulties arise in determining the true relative toxicities of compounds or even derivatives of the same compound if they cannot be applied in the same way. For example, valid comparisons of the true toxicity of the amine and sodium salts with the esters of chlorinated phenoxyacetic acids cannot be made if the esters are applied as oil emulsions and the others in water.

While tests relating to the inhibition of germination or the killing of plants following on spray applications will have a most direct bearing on their possible value in the field, they will be of limited value in the interpretation of these final effects. It has been demonstrated that by varying the composition and physical properties some insight into the parts played by retention and penetration can be gained, yet the part played by movement within the tissues or the toxicity at cell level requires a different approach.

With organisms like yeast or the mould *T. viride* the question of whether movement is extensive or not does not arise, while it will be shown in a later paper that under the experimental conditions employed *L. minor* in some ways behaves like a simple organism. Moreover, since this species is a green plant it provides a bridge between spraying experiments and tests on unicellular organisms.

The basis for interpretation can be extended by studying the reactions of cells or small pieces of tissue, e.g. the comparison of the effects of nitrophenols on the respiration of yeast and leaf disks. Alternatively a number of workers have investigated the effects on the extension growth of roots or root-hairs. Ivens and Blackman (1949) have shown that for seedlings of cereals the degree of inhibition for ethylphenylcarbamate bears a sigmoid relationship to the external concentration. Audus (1949) in his studies of dichlorophenoxyacetic acid and coumarin measured the inhibitory effects on the growth of roots, and although he did not specifically investigate the relationship between dosage and inhibition, inspection of his results points to a sigmoid relationship. Using a somewhat modified technique such a relationship has been found in this laboratory for dichlorophenoxyacetic acid and the growth of the primary root of cress. Wilske and Burström (1950) in their study of thiophenoxyacetic acids and Åberg (1948) and Ekdahl (1948) in their investigations of sodium chlorate have demonstrated that the extension growth of either roots or root hairs was particularly sensitive to small changes in concentration.

On the basis of these results it is evident that for comparative purposes observations on root growth should be of general value. It is, however, once again emphasized that, just as in the other techniques, it is essential before valid comparisons can be made to establish for each type of compound the dosage–response relationships.

For compounds such as the chlorinated phenoxyacetic acids there is already much evidence that the rate and extent of their transport within the plant may govern the degree of injury produced. Thus, in order to determine the importance of this translocation factor, experiments must ultimately be

carried out on whole plants. By localized application or injection and by subsequent observation of the epinastic effects or reductions in growth induced in other parts of the plant, a qualitative assessment of the extent of movement can be attained. It is, however, evident from Fig. 6 that the effects of increasing dose produce different effects in different parts, and in consequence there will be considerable difficulty in interpreting this type of data on a quantitative basis.

Since the amounts of synthetic growth regulator in the tissues which produce biological effects are so small, no satisfactory purely chemical method has yet been devised for their estimation. Under some circumstances the use of a spectrophotometer in the ultra-violet range of wave-length may be of value for relatively micro-amounts (Bandurski, 1947). At present it would seem that success is most likely to come through incorporating isotopes in the molecule and subsequently estimating the content in the tissue samples either by means of a mass spectrometer (e.g. heavy carbon) or by suitable electronic equipment (radioactive isotopes).

Mitchell *et al.* (1947), Wood *et al.* (1947), and Mitchell and Linder (1950) employing radioactive iodine in the molecule have studied the movement of either 2-iodo-3-nitrobenzoic acid or 2:4-dichloro-5-iodophenoxyacetic acid. On the other hand, there are technical difficulties in determining the radioactivity of ground material and similar studies of the rate of penetration and subsequent transport of dichloroiodophenoxyacetic acid have been started in this laboratory using the more precise liquid counter technique. Such a method demands that the compound can be completely extracted from the plant samples, and it is already evident that this in turn presents difficulties. Moreover, it is doubtful whether compounds tagged with iodine behave in the same way as chlorine-substituted phenoxyacetic acids. The alternative approach is to synthesize compounds containing isotopic carbon. Of the radioactive isotopes only C^{14} has a sufficiently long life, but there is the drawback that its activity is low. Probably the most precise data will be forthcoming from the use of material in which heavy carbon has been incorporated not only in the ring but also in the side chain.

This paper has been confined to a discussion of relative toxicity as judged by the effects on whole plants or whole cells, and such a restriction leaves out of account the nature of the toxic action within the cell. Clearly the assessment of toxicity will not be complete until it is known which biochemical reactions or enzyme systems are involved. Where there is evidence that conditions of equilibrium have been established, e.g. frond multiplication of *L. minor* in solutions containing dinitroalkylphenols (Fig. 8*c*) or the growth rate of *T. viride* in the presence of nitro- and chloro-phenols (see p. 21), then—as will be shown in subsequent papers—the thermodynamic considerations of Ferguson (1939) may indicate whether the compound is structurally specific. The analysis of the physical or chemical factors which operate is likely to be much more difficult. In spite of the extensive research in recent years, the mode of action is not yet understood for any phytotoxic compound.

The author wishes to acknowledge the help of Dr. E. K. Woodford and the other members of the Unit of Experimental Agronomy in the preparation of this paper.

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Studies in the Principles of Phytotoxicity

II. EXPERIMENTAL DESIGNS AND TECHNIQUES OF STATISTICAL ANALYSIS FOR THE ASSESSMENT OF TOXICITY

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SUMMARY

Some of the more important statistical methods used in the analysis of experiments concerned with studies of phytotoxicity are described, and are illustrated by data from field and laboratory experiments undertaken within the Department of Agriculture of the University of Oxford. Most attention has been given to a consideration of quantal effects, such as the proportionate mortality. Adjustments to allow for natural mortality or the appearance of additional plants during the course of the experiment are outlined, together with the conditions under which the data should be transformed before analysis. Since the relationship between the proportionate response and some function of the dose or concentration of the toxicant generally follows a normal sigmoid law, the methods of probit analysis are appropriate for precise estimations. In this connexion, the design of experiments is discussed and the calculations involved in such an analysis are illustrated.

In investigations where quantitative measurements are recorded, the dose-response relationship may also be of the normal sigmoid form, so that the data can be treated by a modification of the probit technique. The methods of statistical treatment demanded when the dose-response relationship does not conform to a normal sigmoid are briefly discussed.

I. INTRODUCTION

In the introductory paper of this series Blackman (1951) has discussed the mechanism of phytotoxicity and described the different types of experiment used to investigate various aspects of the problem. This paper considers in rather more detail the layout of these experiments and the methods of analysis appropriate to various types of design. The examples are taken from experiments carried out within the Department of Agriculture by the Agricultural Research Council's Unit of Experimental Agronomy; their use in this paper is purely illustrative, and no attempt is made to interpret the results in terms of physiological action.

Experiments dealing with toxic action may be designed to compare the effects of different poisons or the behaviour of different organisms, or to investigate the effect of variation of one or more of the large number of factors which may control toxicity, such as date and method of application, concentration, and type of solvent. Each experiment, though conforming in general to

one of a small number of types, will show its own peculiarities. These are ignored here, as they will be considered in subsequent papers.

It is possible, however, to distinguish two main types of experiment, described here as 'screening experiments' and 'dose-response experiments'. These are discussed in more detail in the next section.

2. DESIGN OF EXPERIMENTS

i. *Screening experiments.* This expression usually implies the comparison of a range of substances tested against one or more organisms or tissues. It may also be used to describe investigations concerned with the comparison of one or more substances on a wide range of organisms. In this paper it is used in its most general sense to apply to all experiments which are concerned with large response differences obtained by using a range of chemicals, tissues, organisms, or conditions of application.

Because such experiments are designed to investigate major variations in response, which may be either of degree or of type, it is not necessary to use a large number of different doses. The number is usually kept small in order to limit the size of the experiment, and a few levels are chosen to cover most of the appropriate range. The design and analysis of these experiments follow the standard methods of field experimentation, as discussed by Cochran and Cox (1950), Fisher (1951), and Yates (1937). The arrangements which have, to date, proved most useful in herbicidal work are the randomized block design, and various types of plaid design allowing for the planting of crops, and the application of spraying treatments, in long strips. Lattice and other designs have also been used.

ii. *Dose-response experiments.* These are designed to investigate in detail, and to compare, the dose-response relationships of a few treatments. They may be concerned with differences in the chemicals applied or in the plant material treated. More dose levels are required than in screening experiments.

3. THE MEASUREMENT OF TREATMENT AND RESPONSE

Throughout this paper the words 'dose' and 'response' are used to describe many different types of measurement.

In experiments performed in the field the dose is measured as the amount of poison applied per unit area or as the concentration of the herbicidal solution. In the laboratory much greater control over application is possible, and dose may be defined in terms of such variables as the amount of herbicide received by each plant, the leaf area treated, and the time of exposure to the poison.

Responses may be quantitative or quantal. Examples of quantitative responses are, for field experiments, yield of crop plant or number of weeds per plot. In the laboratory more detailed observation is possible, and measurement may be made of rate of root growth, respiration rate, and similar variables.

Quantal responses are obtained when each plant is classified into one of two groups, those showing and those failing to show a particular reaction—in herbicidal work usually death. The response is measured by the proportion of

plants showing the reaction. In laboratory experiments a known number of plants is treated and the number that die is recorded. In the field, estimation of proportion killed is more difficult. The plants on each plot are usually too numerous to be counted; the numbers per plot are therefore estimated before and after treatment, by a random quadrat sampling technique, and the proportionate decrease calculated from these values. Between the pre-spraying and post-spraying counts a considerable time may elapse, during which additional plants may appear, and some of the original plants die from causes other than the poison. In the estimation of proportionate kill, allowance must be made for these changes. This is done by including in each block one or more unsprayed control plots. Pre- and post-spraying counts are made on these, and from them the natural death-rate C is estimated. This may differ from block to block, but seldom varies greatly over the whole experiment. It is calculated as

$$C = \frac{\text{Decrease in numbers over all control plots}}{\text{Total of pre-spraying counts on all control plots}}.$$

Then, if P' is the observed proportionate decrease on any treated plot, the adjusted proportion, P, killed by the poison alone may be estimated by the formula (Finney, 1952, § 26)

$$P = \frac{P'-C}{1-C}. \qquad (1)$$

When, as occasionally happens, more plants are found on the control plots after than before spraying, owing to new germination and emergence, the proportionate increase, C', may be calculated, and the observed proportion P' corrected by the formula

$$P = \frac{P'+C'}{1+C'}. \qquad (2)$$

If C or C' varies greatly between blocks, separate estimates should be made for each block, and the observed results adjusted by the use of the appropriate block control mortality.

The estimation (by sampling methods) of proportion killed and the use, under these circumstances, of formulae (1) and (2) are of necessity only approximate. However, in view of the great variability encountered in the data, the approximations are justified, inasmuch as they enable a complicated experiment to be analysed by standard statistical techniques. Occasionally the approximate results are difficult to handle and interpret in terms of the standard methods. In particular, sampling error and chance fluctuation in the actual numbers killed may produce a situation in which the estimated mortality P' on a treated plot is less than the estimated control mortality C, so that the use of formula (1) then produces a negative value of P. The method used to include such values in the analysis varies with the type of experiment and will be considered in the appropriate sections.

4. THE ANALYSIS OF SCREENING TRIALS

The data from screening trials are treated by the methods of the analysis of variance (Cochran and Cox, 1950; Fisher, 1950, 1951; Yates, 1937). These methods are sufficiently well known to need no more than a passing mention here; the first and last of the above references in particular give examples of layout and analysis for designs of many different types.

Some care is necessary in applying these methods directly to data from herbicidal experiments. The significance tests in the analysis of variance are valid only if the conditions of normality and homogeneity of the residual error are satisfied (Cochran, 1947), and frequently the observed data must be transformed before analysis. Bartlett (1947) and Quenouille (1950, ch. 8) have discussed more fully the transformations outlined below and have described the circumstances in which their use is justified. Where the responses are crop yields, or total weights of weeds, the conditions for the analysis of variance may be assumed satisfied unless inspection of the results suggests otherwise. If, however, the observed values cover a wide range, with some very large values and others very near zero, there is a danger that the variance among plots having low yields will be appreciably smaller than that among those with high yields. This difficulty may be overcome by using the transformation

$$y = \sqrt{x},$$

or, where the results vary greatly,

$$y = \log x,$$

and basing the analysis upon the transformed variate, y.

When the data consist of plant counts, and these are not very variable, they too may be analysed by the same methods without transformation. If the variation is more marked, however, the square-root transformation

$$y = \sqrt{n} \text{ or } y = \sqrt{(n+\tfrac{1}{2})}$$

should be used, the latter being preferable, especially when small numbers are involved. When the variation is very great, the logarithmic transformation

$$y = \log n$$

may be preferable.

For quantal response data, where the observed variable is P, the proportion killed, the observed proportions may be analysed directly provided that all values of P lie between 30 and 70 per cent. Otherwise the angular transformation

$$y = \text{arc sin } \sqrt{P} \qquad (3)$$

is appropriate (Fisher and Yates, 1948, Table XII). If the pre-spraying counts differ greatly from plot to plot, a weighted analysis of variance may be needed. For further refinements in the use of the angular transformation, and for an alternative approach to the problem depending on the use of the probit transformation (section 5), the reader is referred to Jolly (1950).

If small negative values of P, such as might arise from chance fluctuation, are given by formula (1) or (2), they may be taken as zero kills for use in

formula (3). A large negative value, however, suggests that some extraneous factor has affected the results. In such cases the suspect value should be omitted and replaced by the usual missing-plot technique (Cochran and Cox, 1950; Fisher, 1951), after transformation of the remaining values.

5. THE ANALYSIS OF DOSE-RESPONSE EXPERIMENTS

i. *Quantal responses—Probit analysis*

The methods of probit analysis, though extensively used in insecticidal work, have not been widely used in herbicidal testing, and none of the publications concerned with quantal responses in herbicidal investigations (Blackman, 1951; Blackman, Holly, and Roberts, 1949; Fogg, 1948; Ivens and Blackman, 1949) have contained an account of the actual computations involved. It therefore seems desirable to give a brief outline of the method. For a full account, including the mathematical theory, the reader is referred to Finney (1952).

If a single herbicide is applied at a number of different doses to a series of batches of uniform experimental material, and the proportion of plants killed in each batch is calculated and plotted against dose, the points will generally be found to lie on a smooth curve, increasing steadily from zero kill at low doses to 100 per cent. and having the characteristic sigmoid form (Fig. 1). By transforming from the dose to its logarithm the sigmoid may usually be made symmetrical, and frequently assumes the form of the normal sigmoid (Fig. 2), given by

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{(\alpha-5)+\beta x} e^{-\frac{1}{2}u^2}\, du. \qquad (4)$$

This is the form expected if we assume that each individual has a certain critical dose-level which will just kill it, and that, in the whole population of plants, this dose—or rather, its logarithm—is normally distributed. The curve of Fig. 2 never actually attains zero or 100 per cent. kill, but tends steadily towards these values at low and high values of x respectively.

If P is now transformed into a variate Y by the *probit transformation*, defined by the formula

$$P = \frac{1}{\sqrt{2\pi}} \int_{-\infty}^{Y-5} e^{-\frac{1}{2}u^2}\, du, \qquad (5)$$

then the *probit*, Y, must satisfy the relation

$$Y = \alpha + \beta x.$$

The term (-5) in the upper limit in formulae (4) and (5) is introduced to avoid the frequent occurrence of negative values of Y. By plotting probits against log dose, a straight line is obtained (Fig. 3). Tables for the transformation of percentages to probits are given by Finney (1952) and Fisher and Yates

1948, Table IX). The name *dosage*, or *dose metameter*, is given to that function of the dose (usually its logarithm) which gives a straight probit line. The observed points will not be exactly collinear, so that a method is

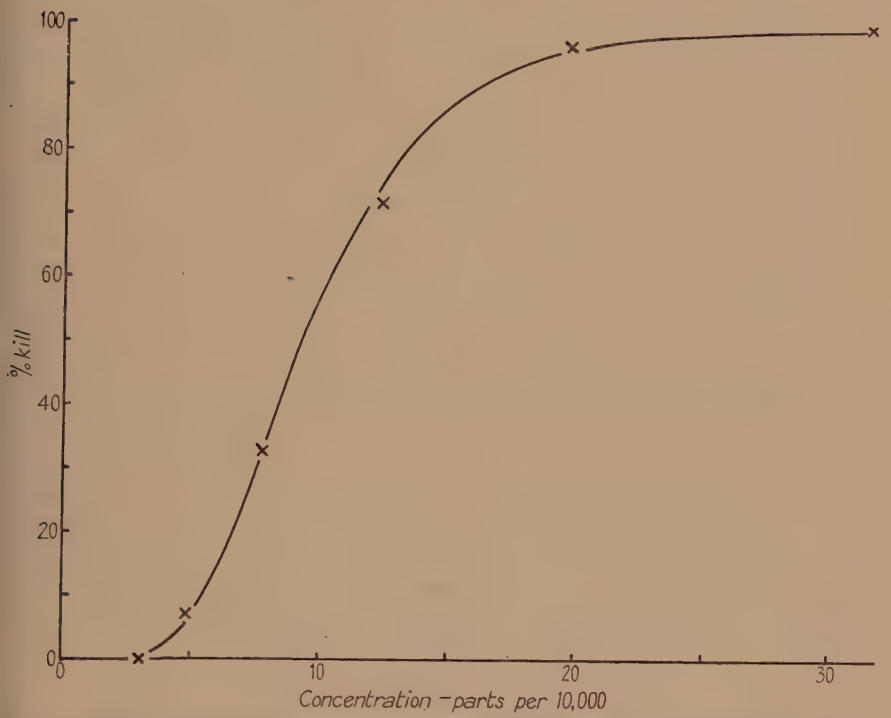

FIG. 1. Example of non-normal sigmoid. Mortality of pea seedlings treated with 2: 4-dinitro-6-*secondary*-butylphenol in a 10% Arachis oil emulsion. Percentage kill plotted against concentration in parts per 10,000. The data for this figure are given in Table I. The curves in this figure and in Fig. 2 are obtained by transforming the probit line fitted to the data in section 5, i.

required for fitting the straight line which best approximates to the observations. An unweighted linear regression is unsuitable, as the amount of information carried by any point depends on the number of subjects tested at that dose; moreover, the mathematical theory shows that the weight of a point based on a given number of individuals is greater in the region of a 50 per cent. kill than at higher or lower values. Equations for estimating α and β, in which each point receives its appropriate weight, may be obtained, but they cannot be solved in terms of elementary functions of the observations. An iterative method is therefore used, as follows:

(i) 'Empirical probits' (obtained by transforming the observed proportions killed) are plotted against dosage, x, and a straight line is drawn by eye to fit the points. This is a first approximation to the best line, and may be represented by the equation

$$Y_{(1)} = a_{(1)} + b_{(1)}x. \qquad (6)$$

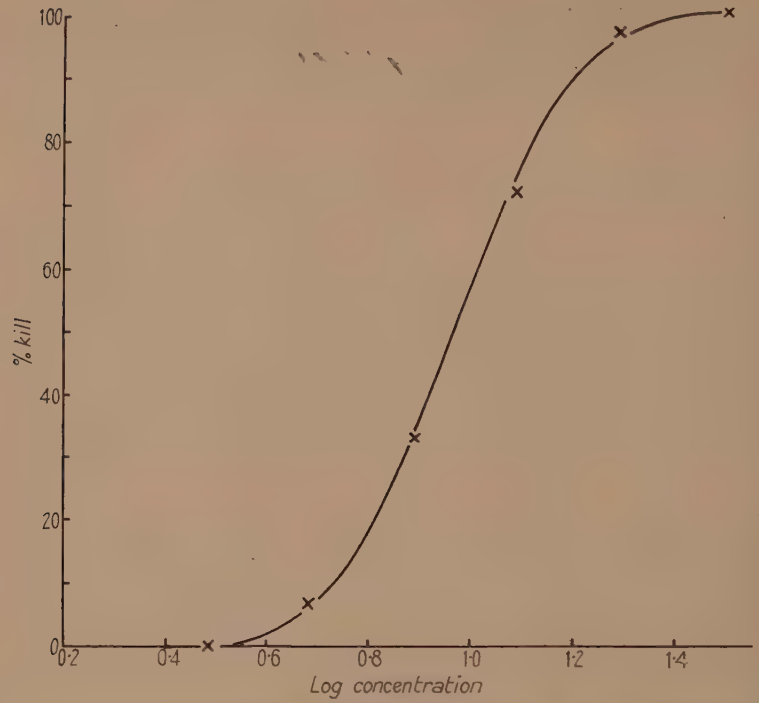

FIG. 2. Example of normal sigmoid. Data of Fig. 1, with percentage kill plotted against log concentration.

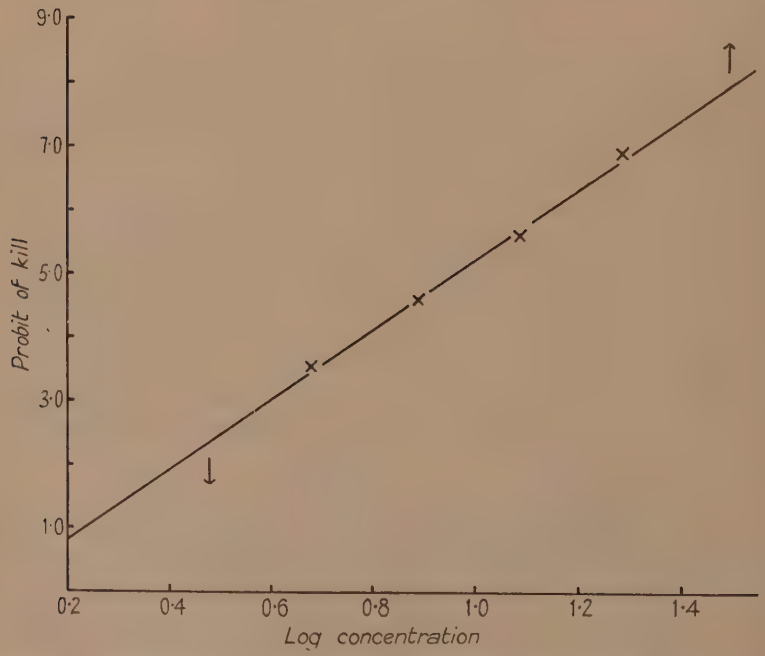

FIG. 3. Example of probit line. Data of Figs. 1 and 2, with probit of percentage kill plotted against log concentration.

(ii) For each dosage used, this line gives the corresponding *provisional probit*, Y. This will not be the same as the empirical probit, for, in general, the points will not lie exactly on the line.

(iii) From tables (Finney, 1952; Fisher and Yates, 1948, Table XI) the *weighting coefficient*, w, and the *working probit*, y, are obtained for each value of Y. Each w is multiplied by the number of subjects treated at the appropriate dose to obtain the *weight*, nw.

(iv) Then if

$$\bar{x} = Snwx/Snw,$$

$$\bar{y} = Snwy/Snw,$$

and

$$b_{(2)} = Snw(x-\bar{x})(y-\bar{y})/Snw(x-\bar{x})^2$$

where S denotes summation over all doses), the weighted regression line of y on x,

$$Y_{(2)} = \bar{y}+b_{(2)}(x-\bar{x}) \qquad (7)$$

is an improved estimate of the probit line. If this line differs considerably from the first approximation (equation (6)) a further improvement may be obtained by recalculating the Y's from (7) and repeating (iii) and (iv) to give a revised version of (7).

The details of the method may be seen in the example (Table I). For each dose, nw and y are tabulated. Columns for nwx and nwy are next constructed and totalled. $Snwx^2$, $Snwxy$, $Snwy^2$ may be calculated on a desk calculator without writing down the separate components. The adjustments for means

$$\frac{(Snwx)^2}{Snw}, \quad \frac{(Snwx)\,(Snwy)}{Snw}, \quad \frac{(Snwy)^2}{Snw}$$

are calculated, and subtracted from $Snwx^2$, $Snwxy$, and $Snwy^2$ to give

$$Snw(x-\bar{x})^2, \; Snw(x-\bar{x})(y-\bar{y}), \text{ and } Snw(y-\bar{y})^2,$$

whence b, $\bar{x}$, and $\bar{y}$ are calculated.

Table I shows the analysis of mortality data of pea seedlings treated with 2 : 4 dinitro-6-*secondary*-butylphenol in an oil emulsion. Empirical probits are calculated and plotted against the logarithm of the concentration in parts per 10,000 (Fig. 3). A provisional line is drawn by eye and the Y values read from it. The calculation than proceeds as previously described. Although zero and 100 per cent. kills give infinite empirical probits, this does not affect the remainder of the calculation, values of Y, w, and y being obtained as for the other points.

The residual sum of squares about the probit line must be considered before standard errors are calculated. This is obtained as

$$Snw(y-\bar{y})^2 - \frac{\{Snw(x-\bar{x})(y-\bar{y})\}^2}{Snw(x-\bar{x})^2}, \qquad (8)$$

and should be tested as χ^2, with degrees of freedom 2 less than the number of doses. Where very high or very low expected kills are obtained from (7), so

Table I

Analysis of mortality among pea seedlings treated with 2:4 dinitro-6-sec.-butylphenol (= HDNBP) *in 10% Arachis oil emulsion*

Conc. parts HDNBP per 10,000	log conc. x	Number treated n	Number killed r	% killed 100 r/n	Empirical probit	Y	nw	y	nwx	nwy
3·0	0·48	40	0	0	$-\infty$	2·3	1·26	1·97	0·605	2·482
4·8	0·68	41	3	7	3·5	3·4	9·74	3·54	6·623	34·480
7·7	0·89	43	14	33	4·6	4·6	25·82	4·56	22·980	117·739
12·3	1·09	36	26	72	5·6	5·8	18·09	5·56	19·718	100·580
19·7	1·29	39	38	97	6·9	6·9	6·02	6·88	7·766	41·418
31·5	1·50	35	35	100	$+\infty$	8·0	0·51	8·30	0·765	4·233
							61·44		58·457	300·932

$\bar{x} = 0{\cdot}9514$ $\bar{y} = 4{\cdot}8980$

$Snwx^2$	$Snwxy$	$Snwy^2$
57·9045	298·8377	1543·1531
55·6188	286·3213	1473·9595
2·2857	12·5164	69·1936
		68·5393
		0·6543 = Residual ($\chi^2_{[4]}$)

$b = 5{\cdot}4760$

$Y = 4{\cdot}8980 + 5{\cdot}4760\,(x - 0{\cdot}9514)$

$= 5{\cdot}476x - 0{\cdot}311$

$V(b) = 0{\cdot}437503$ $\quad V(\bar{y}) = 0{\cdot}016276$

$s_b = 0{\cdot}661$

that the expected number killed or surviving in any group is less than, say, 5, the values of χ^2 obtained from (8) may be inflated, giving a false impression of significance. In this case expected numbers killed and surviving are calculated for each dose, doses are grouped where necessary to eliminate very small expected numbers, and χ^2 calculated from the formula

$$\chi^2 = S\left\{\frac{n(r-n')^2}{n'(n-n')}\right\}, \tag{9}$$

where n = number treated
r = observed number killed
n' = expected number killed
} at any dose or group of doses.

For a more detailed discussion of this method see Finney (1952, § 18).

In Table I the residual, calculated by (8), is 0·66. As the 5 per cent. level for χ^2 with 4 degrees of freedom is 9·5, this is not significantly large, and there is no need to use formula (9), even though some of the expected values are small.

If the χ^2 value is not significantly large, the variances of $\bar{y}$ and b are obtained from the formulae

$$\left.\begin{aligned} V(\bar{y}) &= 1/Snw \\ V(b) &= 1/Snw(x-\bar{x})^2. \end{aligned}\right\} \tag{10}$$

A significantly large value of χ^2 may result from a systematic departure from linearity, such as that illustrated, for quantitative data, in Fig. 7. Such a departure may be difficult to recognize if only a small number of points are available; but, if the data appear non-linear, the assumptions on which the probit analysis is based are invalid, and a more careful investigation of the behaviour of the poison is required. If, however, there is no systematic departure from the probit line, in spite of the occurrence of a significantly large χ^2, the analysis may be assumed valid, the only modification necessary being the multiplication of the variance formulae (10) by a *heterogeneity factor*, equal to χ^2 divided by its degrees of freedom.

In the example, as χ^2 is not significantly large, formulae (10) give

$$V(\bar{y}) = 1/61.44 = 0{\cdot}016276,$$

and

$$V(b) = 1/2.2857 = 0{\cdot}437503;$$

hence, for the standard error of b,

$$s_b = 0{\cdot}661.$$

The slope of the provisional line was 5·58, so that b has been changed by less than one-sixth of its standard error, suggesting that no great improvement can be expected from a further cycle. (In fact, a second cycle increases b by only 0·02, about 3 per cent. of its standard error.)

When control mortality is present, and the results have been adjusted by (1), empirical probits are obtained from the adjusted values. The weighting coefficients w are read from tables (Finney, 1952; Fisher and Yates, 1948,

Table XI_1) giving appropriate values for various levels of control mortality, and the remainder of the calculation is unchanged. If necessary, the iterative procedure can be modified to improve the estimate of control mortality (Finney, 1952, ch. 6). If negative values of P are obtained from (1), they should be included in the calculation, Y and w being obtained as for zero kills. However, working probits are not tabulated for negative P, and must be calculated by methods given by Finney (1952, §§ 16, 27).

A convenient single index of the average level of toxicity of a poison is the *median lethal dose*, or L.D. 50; this is the dose that will, on an average, give 50 per cent. kill. Consideration of (5) shows that a 50 per cent. kill corresponds to a probit value of 5, giving

$$5 = \bar{y}+b(m-\bar{x}),$$

where m is the log L.D. 50, or

$$m = \bar{x}+\frac{5-\bar{y}}{b}. \qquad (11$$

The variance of m is given by

$$V(m) = s_m^2 = \{V(\bar{y})+(m-\bar{x})^2V(b)\}/b^2$$

and, approximately, if the logarithms of the doses have been taken to base 10

$$\text{S.E.(L.D. 50)} = 2{\cdot}3026\times(\text{L.D. 50})\times s_m.$$

These formulae give, for the data of Table I,

$$m = 0{\cdot}9514+\frac{5{\cdot}0000-4{\cdot}8980}{5{\cdot}4760}$$

$$= 0{\cdot}9700$$

$$V(m) = \{0{\cdot}016276+(0{\cdot}0186)^2\times 0{\cdot}437503\}/5{\cdot}4760^2$$

$$= 0{\cdot}00054782$$

$$s_m = 0{\cdot}0234$$

whence L.D. 50 = $9{\cdot}33\pm 0{\cdot}50$ (parts HDNBP per 10,000).

The distribution of m is seriously affected by variations in the value of b, and care should be taken in using its standard error in tests of significance. However, fiducial limits of m may be calculated from the formula

$$m+\frac{g}{1-g}(m-\bar{x})\pm\frac{\tau}{b(1-g)}\sqrt{\{(1-g)V(\bar{y})+(m-\bar{x})^2V(b)\}}, \qquad (12$$

where $g = \tau^2V(b)/b^2$, and τ is the normal deviate corresponding to the desired probability level; e.g. $\tau = 1{\cdot}96$ for 95 per cent. fiducial limits. If the residual χ^2 is significantly large, τ must be replaced by the appropriate t-deviate, with degrees of freedom equal to those of χ^2.

In the example

$$g = (1{\cdot}96^2 \times 0{\cdot}437503)/5{\cdot}4760^2 = 0{\cdot}0560,$$

and 95 per cent. fiducial limits for m are

$$0{\cdot}9239 \text{ and } 1{\cdot}0183,$$

which give, as 95 per cent. fiducial limits for the L.D. 50,

$$8{\cdot}39 \text{ and } 10{\cdot}43.$$

ii. *Comparison of two or more probit lines*

Experiments frequently involve two or more dose-response relationships. For example, they may be designed to compare the effects on a given crop or weed of two different herbicides, the effects of a single compound on two different weeds, or on one weed at different stages of growth, or the effect produced by changing the medium of application.

Blackman (1951, Fig. 5) compares the probit lines for application in oil emulsion and in water of two herbicides, each applied to two plant species. In each case the line corresponding to the oil emulsion has a slope different from that for the water.

The sets of lines obtained in two other experiments are illustrated in Figs. 4 and 5. Fig. 4 shows lines obtained from the effects of three different herbicides (2 : 4-dinitro-6-*secondary*-butylphenol, 3 : 5-dinitro-*o*-cresol, and sodium 2-methyl-4-chlorophenoxyacetate) on linseed grown in pots. The lines for the first two are apparently parallel, but the slope of the third line is considerably different from that of the others. This is a common situation, lines for compounds having similar physiological action on the subject being parallel, and for those having different actions being non-parallel. Fig. 5 shows lines obtained by the application of 2 : 4-dinitro-6-*secondary*-butylphenol to stages of growth of *Stellaria media*. The older plants give a less steep regression line. This is to be expected, as the lower value of b, corresponding to a higher variance in the distribution of critical values, reflects the increase with growth of variability among plants of the same age.

When the probit regression lines obtained from two compounds are parallel, the horizontal distance between the lines, measured on the dosage scale, is constant. This distance, the difference between the dosage of compound 2 required to produce a given kill and the dosage of compound 1 required to produce the same kill, is called the relative dosage value, M_{12}, and is conveniently calculated as the difference between the two log L.D. 50's,

$$M_{12} = m_2 - m_1. \tag{13}$$

The relative potency, ρ_{12}, is defined as the antilogarithm of the relative dosage value:

$$\rho_{12} = 10^{M_{12}} = \frac{(\text{L.D. } 50)_2}{(\text{L.D. } 50)_1}.$$

Whatever the dose of compound 1 which produces a particular kill, a dose

ρ_{12} as large of compound 2 will produce the same kill. In such a case, ρ_{12} is a valuable measure of the difference between the two compounds.

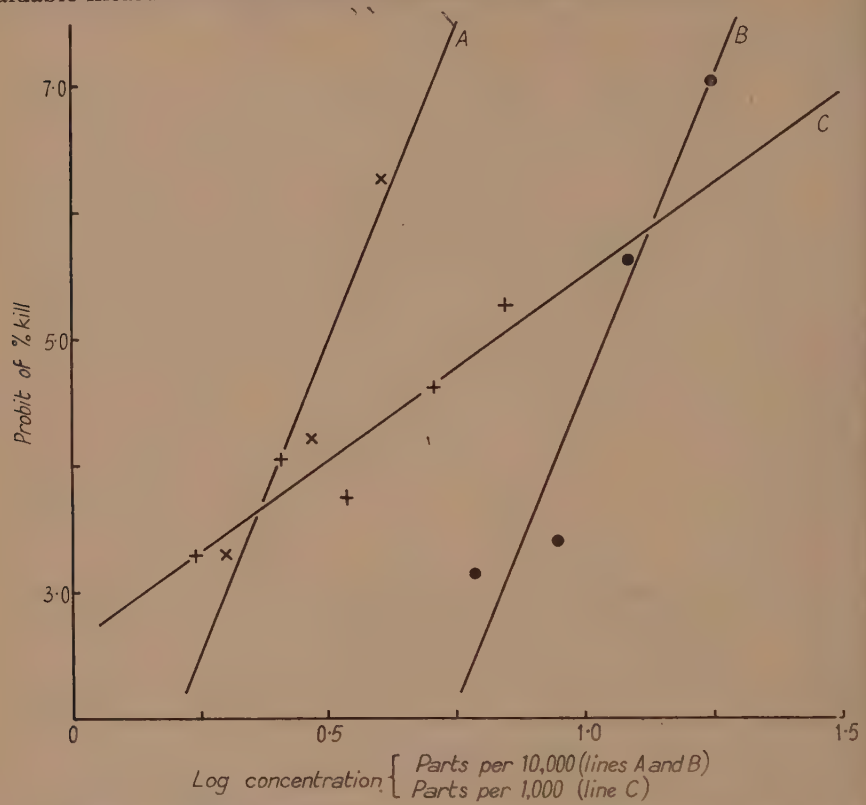

FIG. 4. Effect on linseed grown in pots of 2:4-dinitro-6-*secondary*-butylphenol (line *A*), 3:5-dinitro-*o*-cresol (line *B*), and sodium 2-methyl-4-chlorophenoxyacetate (line *C*). Probit of kill plotted against log concentration in parts per 10,000 (lines *A* and *B*) and parts per 1,000 (line *C*).

When two lines are to be compared, and there is reason to expect parallelism, the data should first be examined for any indication of departure from parallelism. If no such indication appears, the provisional probit lines are drawn parallel. The calculations then proceed independently for the two lines until the calculation of b. As the lines are to be assumed parallel, this must be estimated as

$$b = \frac{S_1 nw(x-\bar{x}_1)(y-\bar{y}_1)+S_2 nw(x-\bar{x}_2)(y-\bar{y}_2)}{S_1 nw(x-\bar{x}_1)^2+S_2 nw(x-\bar{x}_2)^2},$$

where S_1, S_2 are sums over series (1) and (2) only, and $(\bar{x}_1, \bar{y}_1)$, $(\bar{x}_2, \bar{y}_2)$, the means for the two series. For more detail, and for the significance test of deviations from parallelism, see Finney (1952, ch. 5). If the two lines do not differ significantly in slope, their equations are

$$Y_1 = \bar{y}_1 + b(x_1 - \bar{x}_1)$$

and $$Y_2 = \bar{y}_2 + b(x_2 - \bar{x}_2),$$

whence, and from formulae (11) and (13),

$$M_{12} = (\bar{x}_2 - \bar{x}_1) + \frac{\bar{y}_1 - \bar{y}_2}{b}.$$

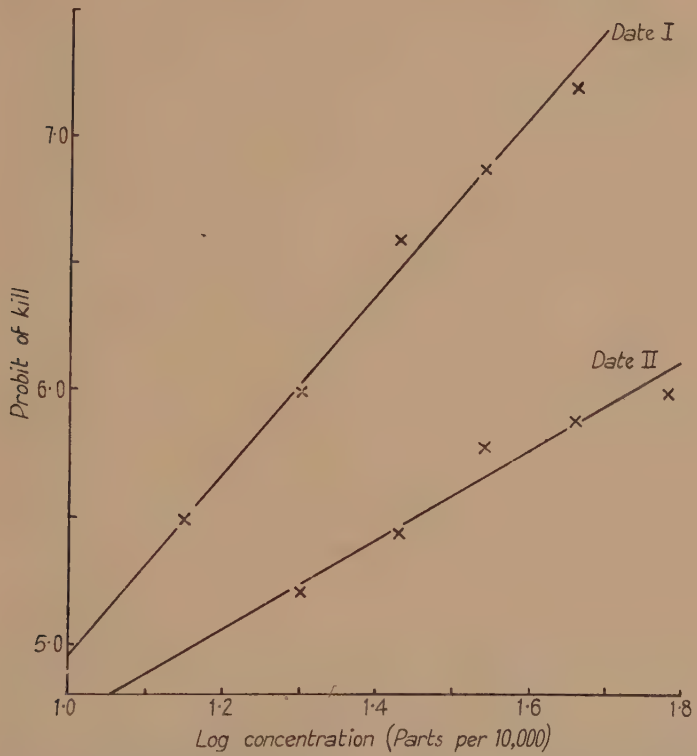

FIG. 5. Mortality among plants of *Stellaria media*, treated with 2:4-dinitro-6-*sec.*-butylphenol at two stages of growth. At date I the plants were at the four-leaf stage. By date II branching was considerable, giving complete ground cover. Probit of kill plotted against log concentration in parts per 10,000.

It can be shown that

$$V(M_{12}) = \frac{1}{b^2}\left\{\frac{1}{S_1 nw} + \frac{1}{S_2 nw} + \frac{(\bar{x}_2 - \bar{x}_1 - M_{12})^2}{S_1 nw(x_1 - \bar{x}_1)^2 + S_2 nw(x_2 - \bar{x}_2)^2}\right\},$$

an expression which must be multiplied by the heterogeneity factor if the combined χ^2 for the two lines is significantly large. An approximate standard error for ρ_{12} may be calculated from the formula

$$\text{S.E.}\,(\rho_{12}) = \rho_{12} \times 2{\cdot}3026 \times \text{S.E.}\,(M_{12}).$$

Fiducial limits to M_{12} may be calculated by a formula similar to (12) (see Finney, 1952, ch. 5) and the antilogarithms of these limits provide fiducial limits to ρ_{12}.

If the probit lines for the two compounds are not parallel, it is still possibl to calculate the log doses of the two compounds required to produce an particular proportionate kill, and hence a 'relative potency' valid for that leve of kill only. There is, however, no longer any single index which will serv to compare the behaviour of the two compounds at all doses.

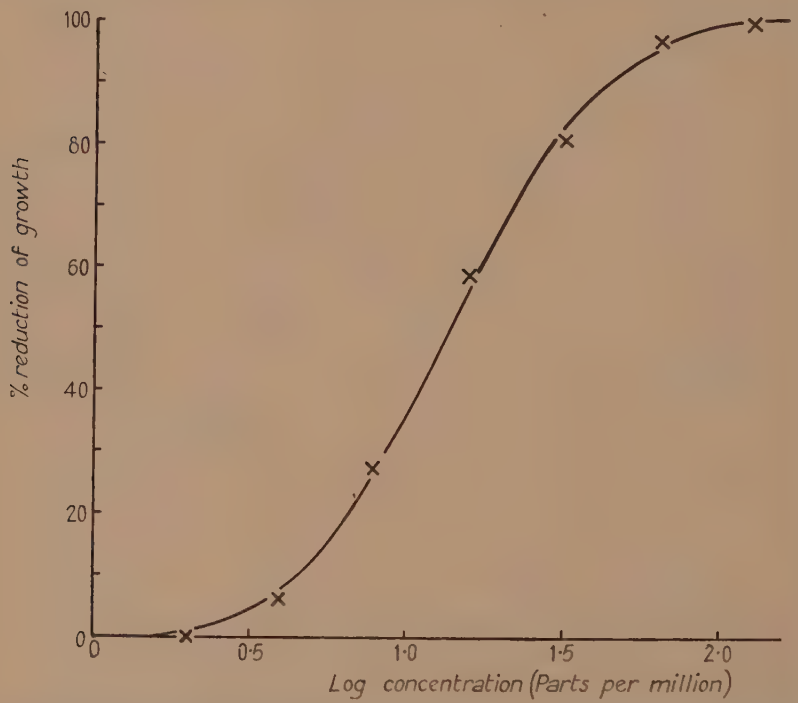

FIG. 6. Reduction of growth of oat roots treated with ethylphenylcarbamate. Reduction as percentage of growth of untreated plants plotted against log concentration in parts per million.

iii. *Mixtures of herbicides*

A number of experiments have been carried out to investigate the dose-response relationships of mixtures of herbicides (Blackman, 1951, Figs. 4*b* 4*c*). The methods of analysis appropriate to experiments of this type are complicated, and, except in certain special cases, still somewhat tentative They are therefore not discussed in this paper. For information on statistical methods the reader is referred to Finney (1952, ch. 8), Plackett and Hewlett (1948), and Hewlett and Plackett (1950).

iv. *Additional dosage variates*

Where two or more dosage variates are used, a simple extension of the probit method is made. For example, if x_1 is the logarithm of the concentration, and x_2 that of time of exposure, we may fit

$$Y = \alpha + \beta_1 (x_1 - \bar{x}_1) + \beta_2 (x_2 - \bar{x}_2).$$

This may be transformed into a single line of the form

$$Y = \alpha' + \beta_1 x_3$$

where $x_3 = \log \{\text{conc.} \times t^{\gamma}\}$.

This method may be extended to any number of dose-variates.

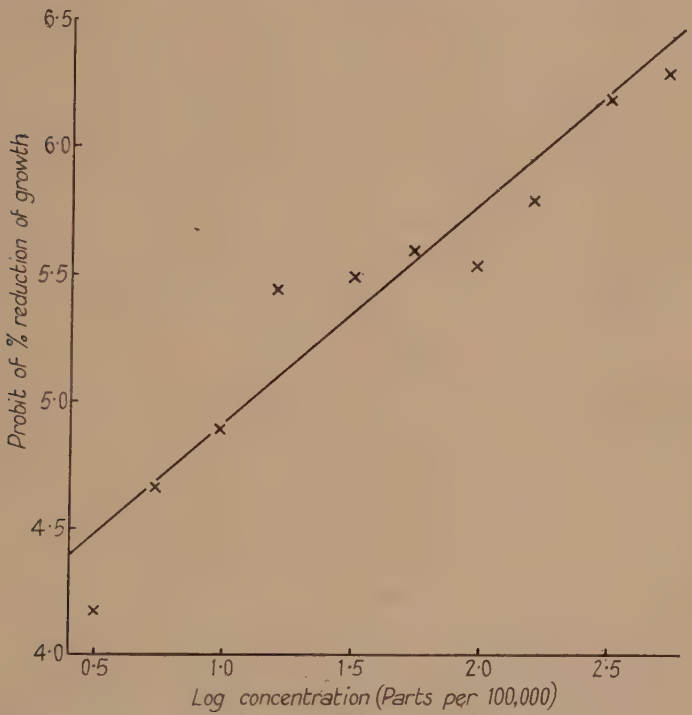

FIG. 7. Reduction of growth of maize roots, treated with sodium chlorate. Reduction expressed as percentage of growth of untreated plants; probit of percentage plotted against log concentration in parts per 100,000.

. Quantitative responses

Where quantitative measures are observed and plotted against dose, curves of various types are obtained. Examples are given by Blackman (1951) and in Figs. 6–8 of the present paper.

In some cases, normal sigmoid curves are obtained by a suitable choice of dose metameter. Fig. 6 shows the percentage reduction of root growth rate in oats, treated with various concentrations of ethyl phenyl carbamate. Other quantitative responses which give normal sigmoids are the reduction in vegetative growth of linseed plants, treated with sodium 2-methyl-4-chloro-phenoxyacetate, and the proportional decrease in growth produced by various fungicides (Simon and Blackman, 1949; Blackman, 1951, Fig. 9).

Such data may be treated by a modification of the probit technique. The weights used earlier are appropriate only for quantal response data and must be replaced by others for quantitative data (Finney, 1952, ch. 10).

The logistic, rather than the normal sigmoid, is frequently used in fitting growth curves of this type. The two curves are so similar that they give results that are, for practical purposes, almost identical.

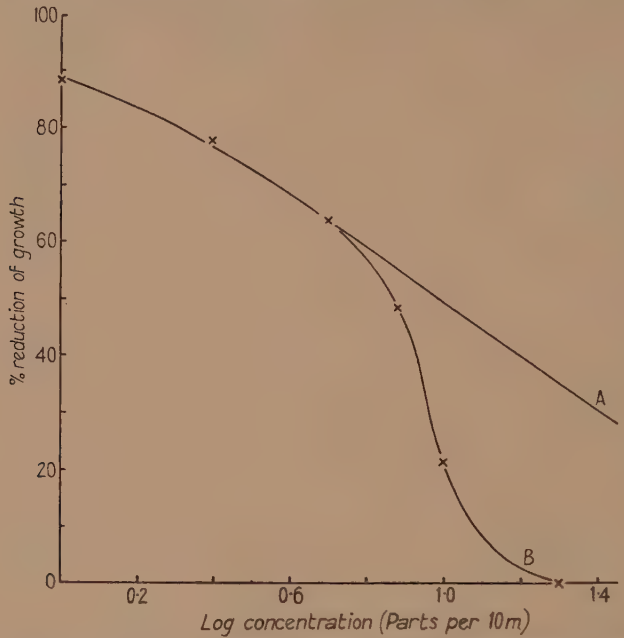

FIG. 8. Reduction in reproduction rate of *Lemna minor*, treated with 2:4-dinitro-*o*-N-butylphenol. Reduction as percentage of control plotted against log concentration in parts per 10 million. Line *A*, though apparently straight, is in fact part of a normal sigmoid fitted to the first three points, and intended to represent inhibitory effects only. Line *B*, drawn free-hand to fit the observed points, departs from *A* at high doses owing to mortality among the parent fronds.

There are, however, many cases in which quantitative data do not conform to the normal sigmoid. For example, the percentage reduction of root growth of maize, treated with sodium chlorate, gives a sigmoid when plotted against log concentration. However, it differs from a normal sigmoid in having a central section, covering a wide range of doses, over which the percentage reduction is practically constant. When plotted on the probit scale, this is indicated by a systematic departure from linearity (Fig. 7).

Blackman (1951, Fig. 7*a*) discusses the effect of three hydrocarbons on bean leaves. When percentage reduction of weight is plotted against volume of hydrocarbon applied, the points, over the range considered, lie on straight lines through the origin. As the reduction approaches 100 per cent., a flattening-out must occur, but these lines will certainly not transform into normal sigmoids. Over this observed range the lines may be satisfactorily fitted by the usual methods of linear regression.

Fig. 8 shows reproduction rates of fronds of *Lemna minor*, plotted, as

percentages of the control reproduction rate, against log concentration of 2:4-dinitro-*o*-N-butylphenol. The points lie on a normal sigmoid for low doses, but at high doses many of the plants die and the curve departs from normality. If enough points were available at doses sufficiently low for the possibility of death to be ignored, a sigmoid representing inhibitive effects only could be fitted, using only those points and ignoring any results obtained at high doses.

The effect of the substituted phenols on the respiration rate of yeast has been discussed by Simon and Beevers (1951) and Blackman (1951, Fig. 10*a*). When the respiration rate, expressed as a proportion of the control rate, is plotted against log concentration of the toxic agent, there is clear indication of stimulation at low concentrations. The curve does not conform to any simple mathematical model, and no routine method of analysis is available. In practice, the curve is drawn by eye and the concentration giving a 50 per cent. reduction of the respiration rate is read off. The same method is used for other results which do not conform to one of the simple models.

This paper has been written in co-operation with the Agricultural Research Council's Unit of Experimental Agronomy, and my thanks are due to Professor G. E. Blackman and other members of the Unit for their advice and criticism. I am particularly indebted to Dr. E. K. Woodford for his help and for his selection of the data cited in the examples. My thanks are also due to Dr. D. J. Finney, Lecturer in the Design and Analysis of Scientific Experiment, for his advice.

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The Polarographic Determination of Phosphate

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SUMMARY

A method is described for estimating polarographically the amount of phosphate in small volumes of liquid, where the phosphorus content lies between 0·5 and 10·5 μg. P/ml. with an error of ±0·2 μg. P/ml. even when chloride, nitrate, and sulphate are present in excess. The method is based on precipitation of uranyl phosphate from uranyl acetate and estimation of the uranyl ion left in solution.

A comparison is made with the colorimetric method of Berenblum and Chain.

A COMPREHENSIVE account of polarographic analysis applied to botanical nvestigation has been given by Riches (1948), but no method was given for hosphate.

In the handbook accompanying the Cambridge Polarograph, used in this vork, an indirect method using uranyl acetate is suggested, which proved omewhat suitable for high phosphate concentrations only. Modification of he method, as given below, proved effective for small quantities of very ilute phosphate solutions.

The phosphorus in the solution to be tested should be converted to ortho-hosphate if not already in that form, and should be present in an amount reater than 0·4 μg./ml. solution. The effect of organic matter being variable, he solution must be free of such matter. In the account given below 5 ml. of olution was always used, but with the polarograph much smaller volumes an be utilized.

The uranyl acetate (analar grade) need not be made up accurately, but eeds to be 0·002M±5 per cent. It is a good practice to make up a fresh olution for each series of analyses, as the solution is changed in some way by ight, so that only a few hours in bright sunlight completely prevents any pre-ipitation of uranyl phosphate. However, a solution kept in a black bottle and t 20° C. showed no change in a month in its ability to form a precipitate. The ffect of light is peculiar in that no change can be detected polarographically n the changed ineffective uranyl acetate. A similar phenomenon is encoun-ered when uranyl is used to separate potassium and sodium in chemical work.

Sodium acetate 0·125 M., pH 4·8–5·5 (preferably pH 4·8) is used as a ground iquid.

Method. Run 5 ml. of phosphate solution into a long narrow test-tube Monax 1·5 cm. diameter are suitable). The solution should not be more acid han pH 4·5. Add 2 ml. of 0·002 M (approximately) uranyl acetate solution. Raise to 70° C. approximately and leave to cool for about 3 hours, avoiding oss of water, which produces a serious error, or, alternatively, leave at room

temperature for 24 hours. The test-tube should be screened from light as much as possible, and left in the dark for the precipitate to settle.

When the precipitate has settled, remove a convenient volume of supernatant liquid and add it to an equal volume of 0·125 M sodium acetate. It is most important not to disturb the precipitate as it redissolves in the sodium acetate, giving rise to large errors. The uranyl-sodium acetate mixture may now be polarographed at a sensitivity of $\frac{1}{3}$. The uranyl ion gives a well-defined easily measured step with a half-wave potential at −0·38 Volts.

To calibrate a capillary 1–5 ml. of $6{\cdot}6 \times 10^{-5}$ M and of $3{\cdot}3 \times 10^{-4}$ M K_3PO_4 (or $NH_4H_2PO_4$) were made up to 5 ml. with distilled water and treated as described above. There should also be at least two blanks (no phosphate) which should give current step waves not differing normally by more than 0·2 mm. in height. The known quantity of phosphate is plotted against the difference between the height of the current step (wave) of the blank, and that of the step in the presence of phosphate. Thus it is that slight variations in the strength of the uranyl acetate are cancelled out. As the uranyl diffusion current varies slightly with pH subsequent analyses should be at the pH of the calibration.

Errors, even in the presence of 0·02 N. nitrate, 0·004 N. chloride, and 0·02 N. sulphate were not consistently greater than the errors inherent in the method, which were rarely greater than ±0·3 μg. P/ml. of orthophosphate solution, and usually less than ±0·2 μg. P/ml. even though step heights were estimated only to the nearest 0·5 mm.

Table I shows figures for a typical calibration, and the negligible effect of the presence of nitrate, chloride, and sulphate.

TABLE I

The Relation between the weight of phosphorus and uranyl current step height (mm.), using $NH_4H_2PO_4$ in solution.

(*a*) 0·0004 N. K_2SO_4 present. (*b*) 0·008 N. $Mn(NO_3)_2$ present. (*c*) 0·0008 N. Ba Cl$_2$ present. (*d*) 0·004 N. Mn $(NO_3)_2$ present.

Weight of phosphorus (μg.) in 1 ml. solution	Polarogram height (mm.) (Uranyl step)	Change in Step height (mm.)
0·00	51·5	0·0
10·33	15·0	36·5
8·26	21·5	30·0
6·20	29·0	22·5
6·20	28·5	23·0 (*a*)
6·20	29·0	22·5 (*b*)
6·20	28·5	23·0 (*c*)
4·13	36·5	15·0
2·07	44·5	7·0
1·64	46·0	5·5
1·64	45·5	6·0 (*d*)
1·24	48·0	3·5
1·24	48·0	3·5 (*a*)
0·82	49·5	2·0
9·41	51·0	0·5
0·00	51·5	0·0

A graph derived from these figures is shown at Fig. 1. In this case $NH_4H_2PO_4$ was used, but shows no significant difference from a similar alibration using K_3PO_4.

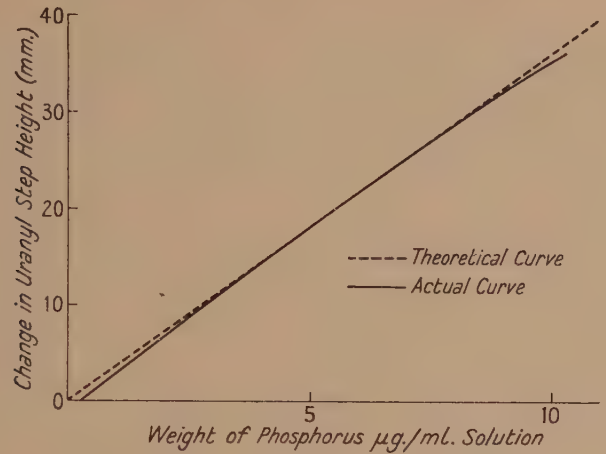

FIG. 1. The Polarographic Determination of Phosphates as Orthophosphate. Graph showing the Relation between the Change in the Uranyl Polarogram and the Orthophosphate in Solution, using $NH_4H_2PO_4$ (Table I).

Reference has been made to the negligible effect of nitrate, chloride, and ulphate. The chief metallic ions which might be present in quantity in a olution of plant ash are calcium, potassium, magnesium, iron, manganese, inc, copper, molybdenum, nickel, and cobalt. Within the pH range given for he ground liquid (4·8 to 5·5) the half-wave potentials of all these metals part from iron are so far removed from uranyl as to give no interference, and ven with iron with tests at pH 4·8 there is no interference.

However, Kolthoff and Lingane (1941) report that the diffusion current of he uranyl ion is greatly dependent on the kind and concentration of salts resent. In a solution of a typical plant ash prepared so that the ortho phos- hate was roughly 0·004 N. the concentration of other ions would be of the order f 0·015 N. Ca; 0·007 N. K; 0·003 N. Mg; 5×10^{-5} N. Fe, Zn, Mn; 5×10^{-6} N. Cu, Mo; 5×10^{-7} N. Co.

It has been found in this method that 0·125 N. Ca produced a 50 per ce nt rror, 0·1 N. K a 4 per cent. error, 0·2 N. Mg a 20 per cent. error, whereas 10^{-3} N. 'e, Zn, Mn, and Co, produced no significant error. Molybdenum has not been xamined. Calcium, magnesium, and potassium in the above tests were in oncentrations approaching closely that of the ground liquid, but at concen- rations of 0·02 N. calcium and potassium and 0·004 N. magnesium, the errors ere insignificant. Copper had the curious effect of increasing the height of the ranyl step, giving a 10 per cent. error when present at 2×10^{-4} N., but no ignificant error at 5×10^{-5} N.

Thus with the dilutions employed interference by complex formation does

not appear to be significant. The effect of complex formation by the uranyl ion with higher concentrations of uranyl acetate has not been investigated.

DISCUSSION

It will be observed from the graph that as the phosphate solution more nearly approaches the uranyl acetate in its equivalent strength, not as much phosphate precipitates as would be expected, so the step height-concentration relation is not linear. This has occurred in all calibrations under these conditions. While the uranyl acetate is in excess the graph continues as a straight line. Hence if it is required to measure without dilution solutions containing, for example, between 6 and 12 μg P/ml., a uranyl acetate solution of 0·004 M should be used, when it will be found the graph continues in a straight line until equivalence is again approached. It appears inadvisable to have more than 15 μg. P/ml., as with the higher concentration of uranyl acetate required there is an interaction with other ions.

The graph also cuts the abscissa at 0·15 μg. P/ml., which appears to indicate that uranyl phosphate in the presence of considerable excess of uranyl acetate is still appreciably soluble at 20° C., the temperature at which the precipitate formed.

To compare the method with that of Berenblum and Chain (1938), the range if 10 ml. of solution were used would be 5–100 μg. as compared with 1–10 μg. P/ml. colorimetrically. However, in the polarographic method no micro technique is necessary if one uses 2 ml. of solution, which extends the range to 1–20 μg. P.

The polarographic technique described is suitable for determination of 5–100 μg. with normal volumes or 1–20μg. if 2–ml. samples are used. This compares with the range of 1–100 μg. using Berenblum and Chain's colorimetric technique, which, however, covers a range as low as 0·1 μg. using micro methods.

The colorimetric method requires only the removal of proteins, whereas in polarographic work the behaviour of unknown organic materials is so unpredictable that no unknown organic material should be present. In this polarographic method, within the limits given no evidence of interference by inorganic ions has been found.

The polarographic method is more pleasant to use than the colorimetric as fewer accurate measurements are required and it uses milder reagents than the 10 N. sulphuric acid and fuming $SnCl_2$; moreover, the vapour of isobutyl alcohol may be undesirable. It appears as reliable as the colorimetric method. It is possible that the error of $\pm 0{\cdot}2\,\mu$g. P/ml. could be reduced. In the calibrations quoted step height was always measured to the nearest 0·5 mm., but with special care many polarographists claim an accuracy of 0·1 mm. It was the intention of the writer to avoid microtechnique and methods requiring great care, and it is remarkable therefore that such reliable results have been obtained so readily with this method.

The method is adequate for the purpose for which it is required in this

aboratory, that is the study of the absorption of substances from relatively ure aqueous sprays by leaves.

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A Note on the Presence of β-Amylase in Moulds

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SUMMARY

Evidence is given for the presence, in the mould *Mucor Rouxianus* and in several *Rhizopus* species, of an amylolytic enzyme producing glucose directly from starch. This glucosidase probably possesses a wide specificity range and attacks maltose as well. It does not destroy the iodine reaction of starch, but as a rule it is accompanied by another amylase which does destroy this reaction. Of the mould amylases investigated, *Mucor*-amylase contained the highest proportion of the glucosidase.

INTRODUCTION

EVER since Giesberger's work (1934) on *Aspergillus*-amalyse, the problem the occurrence of β-amylase (saccharogenic amylase) in moulds has been much debated issue. Giesberger himself, in a later communication (193 doubted the validity of his original findings. During the war, however, Le pold and Starbanow (1943) came to the conclusion that a saccharogenic am lase must be present, at least during certain stages of the life-cycle, in *Rhizop japonicus*, a mould which is also known as *Amylomyces* β and which has fou wide application in industry. Extracts of this organism, when allowed to a upon starch, showed different pH optima for dextrinization and sacchari tion, viz. for the former at pH 5·0 and for the latter at pH 4·0. Moreover, t shape of the pH-activity curves was different for the two processes. In suppo of their preliminary conclusion that a β-amylase was responsible for the great part of the saccharification and an α-amylase for the dextrinization, inactiv tion experiments were carried out. In regard to low pH, the dextrinizing pow turned out to be much more susceptible than the saccharifying power, where the reverse was true in regard to thermal influences. These results, like tho of diffusion experiments with extracts of the mould on starch-gelatin plat according to Wijsman (1889, 1890), are in excellent agreement with what known about the behaviour of malt α- and β-amylase. However (as Gie berger already found), the results of a Wijsman test have to be judged wi extreme caution. Furthermore, it seems dangerous to conclude that a com bination of α and β amylase is present in any given material merely on t basis of the relationship between saccharification and dextrinization, as lo as these conceptions remain as ill defined as they are now; a saccharogen amylase that would, for example, produce glucose from starch without inte mediate formation of maltose could not be correctly identified with, f example, malt β-amylase. Considerations like these must be the reason w

cognized experts on starch like Badenhuizen (1949) have not given Leopold ıd Starbanow's findings the attention which they undoubtedly deserve. ıpanese work on *Rhizopus*-amylases (Asai and Matsumoto, 1942), which has so furnished evidence for the occurrence of both saccharogenic and dex-inogenic amylase, has likewise been neglected, probably because it has only ry recently become more widely known (1951). It is difficult to evaluate ecause details are lacking in the short abstract available.

STATEMENT OF THE PROBLEM

The purpose of the present paper is to give unambiguous evidence for the resence or absence of β-amylase in *Rhizopus* and *Mucor*. Such evidence can e given by considering the characteristic ability of β-amylases to produce a nsiderable proportion (64 per cent.) of maltose without destroying the dine reaction of the substrate, soluble potato starch. The maltose has to be entified and assayed by very specific methods such as fermentation or filter-ıper chromatography, and not by measurements of the reducing power one, since these are very misleading.

MATERIALS AND METHODS

Moulds

The following species and strains were investigated: *Rhizopus japonicus* uill. = *Amylomyces* β Boidin; *Mucor Rouxianus* (Calmette) Wehmer; *hizopus Delemar* (Boid.) Wehm. et Hanz No. 4858, and *Rhizopus Delemar* o. 9374. They were kindly furnished by the Centraal Bureau voor Schimmel-ltures, Baarn, Holland, director Professor Dr. Johanna Westerdijk. All ese organisms are used in industry.

Culture-methods

Both culture in liquid media and on plates was practised. Saboureaud edia generally gave excellent growth, but since they led to very early forma-n of spores, they were abandoned in favour of malt and maize media. Malt edia were of the conventional type, maize media were prepared as follows: o g. of coarsely ground maize meal was vigorously stirred with 1 litre of ater and a pinch of salt for about an hour at 55°–60° C. After that the tem-rature was gradually raised so that a thick paste was formed; this was drolysed by means of saliva during the night, at room temperature and H 6·5, with addition of a small quantity of chloroform and some water to ake the paste more manageable. Next day the mixture was strained by press-g it through cloth, the filtrate was boiled, filtered, and finally sterilized at a H not exceeding 6·5 to avoid caramelization. The moulds were grown in tton-plugged, sterile 300-ml. Erlenmeyer flasks containing a shallow layer this fluid, without any further measures; temperature 30° C. For *Rhizopus* culture period of 2–3 days usually produced sufficient mycelium for the periments without giving rise to spore-formation. *Mucor* required a longer riod; however, when agar was added to the maize medium and the moulds

were grown on plates, 2–3 days proved sufficient for all the moulds investigated. A considerable drop in pH was always observed in the liquid medi but as this obviously did not interfere significantly with amylase productio no buffer was added.

c. Extraction of the amylases

The mycelial mats were removed from the flasks or the plates, washe thoroughly with tap-water, and ground in a mortar with quartz sand and small amount of acetate buffer. In some experiments the pH of the buffer w 4·0, in others 3·8. After centrifugation and filtration the amylases were prec pitated in 80 per cent. alcohol, sucked dry, washed with absolute alcohol an acetone, and dried on filter-paper at a temperature of 30° C. In this state th precipitates could be stored at room temperature for several days at least with out losing much of their activity. Wijsman plate tests showed that they d contain two enzymes, one (*a*) destroying the iodine reaction of starch, th other (*b*) producing a violet-staining substance and diffusing more rapid than *a*. In the case of *Mucor b* was found almost pure. The life-stage of th moulds turned out to be important with respect to the production of amylase In agreement with Leopold and Starbanow (1943) it was found that the greate amount of *b* is present immediately before spore-formation. *Rhizopus Deleme* No. 9374 usually contained less *b* than *Rh. Delemar* No. 4858; however, it doubtful whether this difference is really significant.

d. Hydrolysis of starch by the mould amylases and estimation of activity

Mixtures were prepared containing soluble starch, acetate buffer pH 4, an a small amount of the enzymatic powder, the initial starch concentration bein 1·6 per cent. These mixtures were incubated at 30° C. with a small amount chloroform. At regular intervals small samples were boiled to eliminate th disinfectant, which has a reducing power of its own, and, after cooling an making up to volume, the samples were checked for iodine-coloration an reducing power; also, drops were subjected to analysis by filter-paper chroma tography. For quantitative estimation of the sugars formed, the micr fermentation method of van Lutsenburg Maas and van Iterson (1915) w used throughout. The fluids that were subjected to fermentation were obtaine as follows: to samples withdrawn from the digestion-mixtures alcohol w added to give a final concentration of 80 per cent. in order to precipitate de trins and undigested starch; the filtrate was evaporated on a steam-bath an redissolved in a quantity of a 1 per cent. fructose solution equal in volume t the original sample. Blanks were run by fermenting this fructose solutio itself. The yeasts employed were *Candida monosa*, able to ferment on hexose monosaccharides, and *Saccharomyces uvarum*, able to ferment hexo monosaccharides, sucrose, and maltose, but (in marked contrast to *Sacchar myces cerevisiae*) apparently possessing a certain discriminatory power toward the lower dextrins (Smits van Waesberghe, 1941).

RESULTS

In all four moulds investigated it soon became clear from the filter-paper hromatography experiments (Fig. 1) that glucose was the only visible end-roduct of starch degradation at the pH employed (4·0). Therefore it seemed

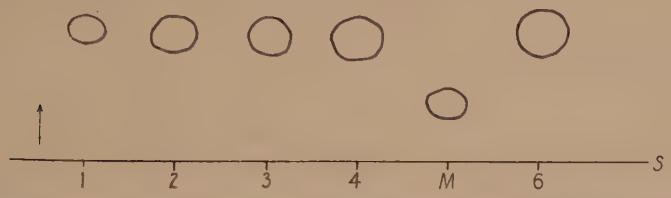

FIG. 1. Lower part of filter-paper chromatogram, produced with drops of fluid taken from one starch-mould enzyme digest on consecutive days (1, 2, 3, &c.). pH of the digests 4·0, temperature 30° C.; enzyme from *Rhizopus japonicus*. Chromatogram run with butanol-acetic-acid-water, ascension of front as indicated by arrow; development with *m*-phenylene-diamine-HCl. *M*: a blank with a drop of maltose solution produced a spot much closer to the start-line *S* than the other drops. The R_F-value of the non-maltose spots corresponds with glucose.

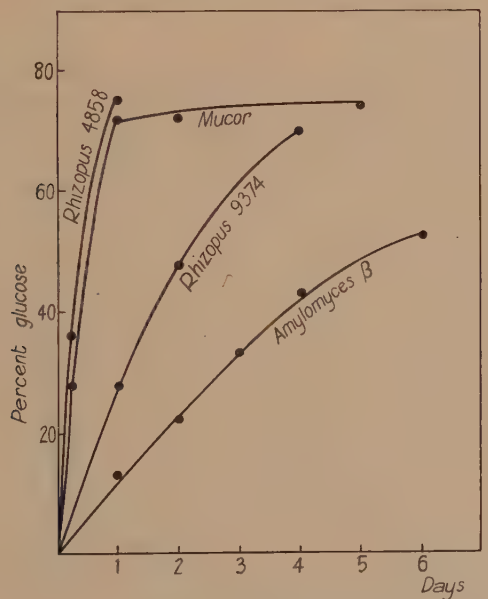

FIG. 2 (cf. Table I). Formation of glucose from 1 per cent. starch by enzymes from *Mucor* and *Rhizopus*, at pH 4·0. Every point corresponds to a hydrolysis-stage where the iodine reaction was still blue, or at least violet, the only exception being the highest point for *Rhizopus* 4858, for which a greenish iodine reaction was found.

afe to make the calculations even for those fermentation experiments where nly *Saccharomyces uvarum* was used on this basis. Fig. 2 and Table I sum-narize the results obtained. Even at very high sugar concentrations (around

TABLE I (cf. Fig. 2)

Formation of glucose from 1 *per cent. starch by enzymes from* Mucor *and* Rhizopus, *at pH* 4·0

Organism	Days of incubation of digest							
	0·25	1	2	2·125	3	4	5	6
Rhizopus japonicus (= Amylomyces β)	—	0·13	0·22	—	0·33	0·43	—	0·525
Rhizopus Delemar 9374	—	0·275	0·48	—	—	0·70	—	—
Rhizopus Delemar 4858	0·36	0·75	—	—	—	—	—	—
Mucor Rouxianus	0·28	0·72	—	0·72	—	—	0·74	—

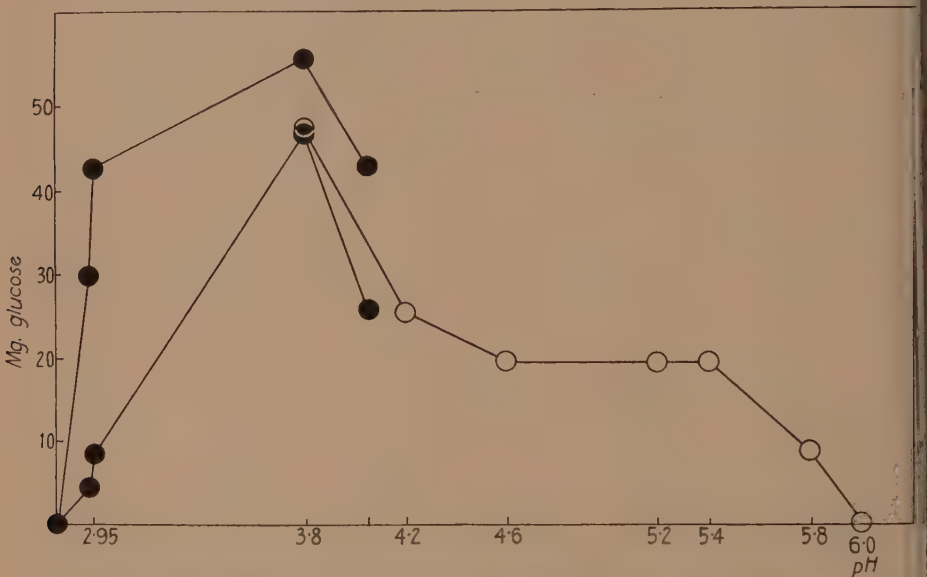

FIG. 3. Optimal pH for *Rhizopus* glucosidase. Substrate: maltose. Black circles: glycine buffer; open circles: acetate buffer. The upper line represents the results of a 5-day experiment, the lower lines give those for a 2-day experiment. The coincidence of the black and open circles at pH 3·8 must be fortuitous, since the experimental conditions were not entirely the same for the two sets of experiments.

70 per cent. of glucose) a blue or blue-violet iodine reaction persists, in marked contrast to the result of experiments with saliva (α-amylase), where only 15 per cent. of fermentable sugar is found when the iodine reaction has become dark brown. However, as stated earlier, this evidence alone is insufficient to confirm the presence of a β-amylase. A system β-amylase-maltase could indeed be active, but the presence of a special amylase hydrolysing starch immediately to glucose had also to be reckoned with. Experiments on the activity towards maltose of extracts from the enzymatic powders led to the conclusion that a maltase (α-glucosidase) with a pH optimum of about 3·8 was active (Fig. 3). Literature data for the pH optima of mould maltases are:

4–4·5 for takamaltase (Leibowitz and Mechlinski, 1925) and 4·2–4·6 for *Aspergillus* maltase (Hofmann, 1935). The maltase of *Rhizopus* and *Mucor* is different, then, from other maltases; the above-mentioned possibility that it might also have a different specificity range, and might attack starch *directly*, did not seem far-fetched. To investigate this point the mould enzymes were allowed to act upon starch at a pH value (6·8) far from the optimum for maltose hydrolysis (pH 3·8) but well within the activity range of true β-amylase from, for example, barley malt. In these experiments, starch breakdown (as apparent from reducing values) proceeded much more slowly than at pH 4. Paper chromatography applied to the digests never revealed the presence of maltose; glucose, however, was always found—in small quantities in fresh digests, in larger amounts in older ones. At pH 2·95, where true β-amylase is not very active, the *Rhizopus* and *Mucor* enzymes still broke down starch rather rapidly, and again glucose was the only free sugar formed. Even when glycerol, a well-known inhibitor of 'true' maltase which does not profoundly influence amylase, was added to neutral digests in concentrations up to 10 per cent. (by volume), we were unable to detect any maltose; yet glucose was found in abundance.

Conclusion

Though, admittedly, all the results here described may be due to the presence in *Rhizopus* and *Mucor* of an enzyme mixture in which a 'true', very specific maltase far outweighs a 'true' β-amylase, it is much more likely that one enzyme, an α-glucosidase with a wide specificity range and therefore able to attack starch, is responsible for the phenomena observed. The action of this glucosidase seems to be restricted to α–1–4 linkages since (in starch) hydrolysis apparently comes to an end as soon as about 70 per cent. of glucose has been formed (cf. Fig. 2); it is quite possible that the 1–6 linkages of the branching points in the amylopectin molecules form an insurmountable obstacle here. In its behaviour, then, this enzyme resembles malt β-amylase. However, since no maltose is produced, it would be wrong to identify it with the latter enzyme.

ACKNOWLEDGEMENTS

The writer wishes to express his sincere gratitude towards the Elizabeth Thompson Science Fund for generous help in equipment, and to Mr. H. E. Latuasan for technical assistance.

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NOTE ADDED IN PROOF

More experiments with somewhat purified enzymes have revealed the fact that the starch-hydrolysis curves usually do not level off as abruptly as the *Mucor* curve in our Fig. 2. An iodine-reaction may persist in stages of hydrolysis corresponding to over 70 per cent. of theoretical glucose. Therefore, it is not at all certain that the action of the glucosidase described is stopped by α-1, 6 linkages. But this can be interpreted as even stronger evidence for the special nature of the enzyme, different from β-amylase.

The Influence of Internal Ion Concentration on Potassium Accumulation and Salt Respiration of Red Beet Root Tissue

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(*Received 20 July 1951*)

SUMMARY

It is shown that during a period of washing in aerated distilled water disks of Red Beet root tissue acquire the capacity to absorb potassium rapidly. Subsequently the rate of accumulation of this ion is closely related to the internal salt-content of the material. On the other hand, whilst the level of salt respiration increases during the preliminary washing, it is not influenced significantly by the internal potassium concentration. The implications of these observations are discussed in relation to a possible mechanism of mineral salt absorption.

INTRODUCTION

THIS investigation forms part of a more extensive research programme being conducted in this laboratory to examine further the interrelationship between mineral salt absorption and respiration. Although it seems evident that since ion accumulation against a concentration gradient must be an energy-consuming process, it should be intimately involved with at least some component of respiration, the establishment of any direct correlation between the two has for a long time evaded investigators in this field (Hoagland and Broyer, 1936; Steward, 1935, 1937). The experiments of Lundegårdh and Burström (1933, 1935), however, indicated a relationship in root tissue between the absorption of anions and a component of respiration which is relatively very sensitive to cyanide poisoning, and led to the proposal of an hypothesis to explain ion absorption by an electron-transfer mechanism involving cytochrome. This hypothesis has received support from other investigators, notably Robertson and his collaborators working with disks of storage tissue (Robertson, 1941, 1944; Robertson and Turner, 1944; Robertson and Wilkins, 1948). Robertson's work indicated that an effect of salt on the respiration rate of disks of carrot tissue is only clearly demonstrated when the tissue has previously been washed for several days in distilled water, and the same also appears to be the case with beet root disks. Now it is well established (Stiles and Skelding, 1940) that freshly cut disks of storage tissue absorb mineral salts from nutrient solutions only slowly at first if at all, and that often ions are exuded for many hours before active absorption begins. There appears to be the possibility of a connexion between the low rate of absorption and feeble salt respiration here, and it was to investigate this that the present work was initiated. At an early stage interest was focused on the influence of internal salt concentration on absorption and respiration, since this is one of the factors which alters during

washing of the tissue. There appears to be no *a priori* reason why salt respiration should be influenced by the amount of salt already accumulated, but on the other hand internal salt concentration appears to affect profoundly the process of absorption. Hoagland and Broyer (1936) and Stiles and Skelding (1940 *a*) have shown that when the general salt content of a tissue is low, accumulation of ions occurs more rapidly, at least at first, than it does when the internal salt concentration is high. Humphries (1950) has obtained evidence that in some circumstances the absorption of a particular ion, for example, phosphate or nitrate, may be determined, not by the total salt-content, but by the concentration of the particular ion in question. Using quite different research material, namely, cells of *Valonia* and *Halicystis*, Jacques (1938) has demonstrated that if the concentration of salt in the sap is maintained artificially at a low level, absorption occurs more rapidly than it does if the sap concentration is allowed to attain a high level.

The importance of internal salt concentration on potassium absorption by well-washed disks of beet root tissue has been confirmed here, but this factor alone appears to be insufficient to explain the slow rates of absorption observed with freshly cut disks. Moreover, although salt respiration increases during the washing treatment, it is not influenced significantly by the internal salt concentration in well-washed tissue. It is the purpose of this paper to record these observations, and discuss them in relation to the Lundegårdh hypothesis of a salt absorption mechanism.

EXPERIMENTAL MATERIALS AND METHODS

Disks of Red Beet root tissue were used in this investigation for a number of reasons. They will remain healthy for several weeks in aerated distilled water, and under suitable conditions will absorb mineral salts rapidly. In addition these disks do not grow by callus formation during the course of an experiment, and in the absence of actively dividing cells they remain in a more uniform metabolic state. Since the disks do not increase in volume by cell enlargement either after about 24 hours immersion in the salt solution, the influence of accumulation on the internal concentration of salt is more pronounced, and the effect of this factor on the rate of absorption more easily indicated. The disks were cut by means of a hand microtome to a standard size, 7·5 mm. diameter and 0·75 mm. in thickness, and then washed several times in distilled water to remove damaged cell debris. They were then transferred to 250 ml. of glass distilled water maintained at 25° C. in a 500 ml. filtering flask. The liquid was changed after 1 hour, 2 hours, 3 hours, and thereafter daily until the end of the experimental period. The disks were aerated continuously by a rapid air-stream bubbling through the liquid and under these conditions they usually showed no signs of bacterial contamination such as loss of turgidity, or exudation of pigment, nor did the external medium normally become turbid. Occasional groups of disks which became infected were discarded. After the disks had been washed in this way for varying

periods of time, usually a few hours, 4 days, or 8 days, replicate samples of 20 disks were taken and placed in 4 ml. of the mineral salt solution in 50-ml. wide-mouthed conical flasks which were closed with rubber stoppers each carrying two glass capillary tubes through which the internal atmosphere over the solution was maintained in equilibrium with the external air. To facilitate aeration the flasks were mounted in a mechanical shaking apparatus, which continuously agitated the solutions and disks throughout the experimental period. Preliminary experiments were conducted to determine the necessary rate of shaking, such that aeration was not a limiting factor in salt absorption. The disks were kept at 25° C. during agitation by immersion of the flasks in a temperature-controlled water-bath.

Potassium absorption

After 24 hours the flasks were removed from the shaking apparatus and the liquid from each decanted into a 50-ml. volumetric flask. The disks were then washed several times with small quantities of distilled water, which were added to the decanted solutions, and each solution was finally made up to 50 ml. with more distilled water. Potassium determinations were made on these diluted solutions by means of flame photometry, and absorption by the disks in 24 hours calculated. Meanwhile the tissue was immersed in fresh potassium chloride solution, and shaken for a further 24 hours, after which another potassium determination was made. This procedure was repeated for several days until absorption ceased.

TABLE I

Potassium content of disks after immersion for 8 days in potassium chloride solution at 25° C. (A) determined by analysis of the tissue at the end of the experimental period; (B) by analysis of the medium at intervals; (C) the initial content determined as the difference between (A) and (B); (D) initial content found by analysis of the tissue immediately after cutting

	Micrograms per disk			
	I	II	III	IV
A	5,400	5,660	5,560	5,420
B	4,220	4,540	4,560	4,610
C	1,180	1,120	1,000	810
D	1,090	1,160	940	890

Determinations of the internal potassium content of the disks were also made during the preliminary washing period and at intervals during absorption. This was done by taking groups of 20 or 40 disks and heating them in 10 ml. of distilled water at 100° C. for 30 minutes. Preliminary experiments showed that more prolonged treatment in this way did not increase the amount of potassium extracted. The solutions were then decanted into 50 ml. volumetric flasks, and the disks washed several times with small quantities of distilled water, the washings being added to the flasks, and the whole made up to constant volume with more distilled water. The potassium content of these

solutions was then determined with the flame photometer, and from the differences observed absorption by the disks at each interval was calculated. These figures were compared with those obtained by analysis of the external medium, and usually there was agreement with an error of less than 10 per cent. If this was not the case the data were rejected and the experiment was repeated. Some of the determinations made of the potassium content of four replicate groups of disks immediately after cutting, and after immersion for 8 days in 0·02 M. KCl, are shown in Table I.

Fresh-weight

In order to estimate the internal potassium concentration of the disks during an experiment it was necessary to make determinations of the volume occupied by the tissue at intervals. This was most conveniently done by weighing groups of 40 or 80 disks taken from the solutions and dried superficially by placing between sheets of filter-paper under slight pressure for 1 minute. On estimating the internal potassium concentration from the data obtained, it was assumed that the salt present is distributed uniformally throughout the tissue. Since no account was taken of the volume occupied by intercellular spaces and cell walls, where after washing the potassium concentration is likely to be low, it is probable that the figures obtained represent minimum values for the actual potassium concentration in the protoplast and vacuole. It is impossible in this connexion to distinguish here between the salt concentrations in the protoplast and vacuole, which may well be different, but it is not felt that any serious error is introduced in this case by assuming that they are the same.

Respiration

Determinations of the rate of oxygen uptake under different experimental conditions were made with a Warburg apparatus. Groups of 20 disks were taken, either after a period of washing before immersion in the salt solution or following a period of absorption. In the latter case, the disks were washed in several changes of distilled water before introduction to the Warburg flasks. The tissue was suspended in 3·8 ml. of distilled water in the flasks which were then immersed in a thermostat at 25° C. After an equilibration period of ½ hour, measurements of oxygen uptake were made at 15-minute intervals for 1 hour. Then, if the effect of salt on respiration was to be examined, 0·2 ml. of potassium chloride solution at a concentration of 0·4 M. was added from a side-arm, and the absorption of oxygen determined at 15-minute intervals again for 2 hours. The effect of potassium cyanide on the rate of respiration was determined when required, by stopping the experiment temporarily and adding the required amount of potassium cyanide solution to the liquid in contact with the disks. At the same time the caustic potash in the centre well of the flasks was replaced by a fresh caustic potash solution containing the required concentration of potassium cyanide to prevent absorption of cyanide from the medium in which the tissue was suspended (Krebs, 1935). The

flasks were then re-attached to the manometers, and after equilibration, further oxygen absorption determinations were made.

EXPERIMENTAL RESULTS

Fresh-weight

The data shown in Table II and Fig. 1 indicate that at 25° C. freshly cut disks increase rapidly in volume during 24 hours following immersion in aerated distilled water, under the conditions outlined. Subsequently there is no

TABLE II

Fresh-weights (milligrams per disk) during aeration at 25° C. for 8 days in (A) distilled water, (B) 0·02 M. potassium chloride solution

	A			B		
Days	Replicates		Mean	Replicates		Mean
0	35·50	36·40	35·95	35·55	36·10	35·80
1	38·80	37·90	38·35	37·80	38·30	38·05
2	38·55	38·75	38·65	39·35	38·45	38·90
3	38·35	38·65	38·50	38·85	38·75	38·80
4	38·60	38·45	38·55	38·90	39·30	39·10
5	39·05	38·85	38·95	38·85	38·55	38·70
6	38·70	38·20	38·45	38·35	38·85	38·60
7	38·50	38·90	38·70	39·25	38·15	38·70
8	38·45	38·65	38·55	39·20	38·70	38·95

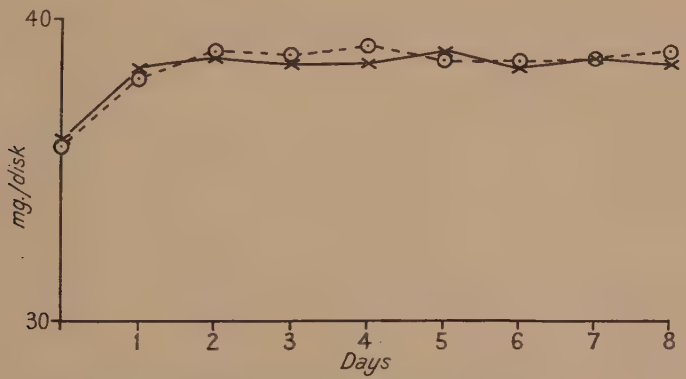

FIG. 1. Fresh-weight of disks during aeration at 25° C. in (*a*) distilled water (—×—), (*b*) 0·02 M. KCl (- - -⊙- - -).

gnificant change in weight for as long as 8 days after cutting. The results also now the disks behave similarly when they are placed for 8 days in 0·02 M. otassium chloride solution at 25° C. in small flasks in the shaking apparatus.

nternal potassium concentration

The changes in the internal potassium concentrations of the disks at inter- ls following a period of washing, and during absorption of salt from a solu- on of potassium chloride at a concentration of 0·02 M., are indicated in able III and Fig. 2. It may be observed that the concentration in freshly cut

TABLE III

Internal potassium concentration (M) of disks at 24-hour intervals during aeration in 0·02 M. potassium chloride solution at 25° C., after preliminary washing in aerated distilled water for (A) a few hours, (B) 4 days, (C) 8 days at 25° C. and for (D) 4 days, (E) 8 days, (F) 12 days, at room temperature (about 14–17° C.)

Days after cutting	(A)	(B)	(C)	(D)	(E)	(F)
0	0·042	—	—	—	—	—
1	0·046	—	—	—	—	—
2	0·057	—	—	—	—	—
3	0·064	—	—	—	—	—
4	0·080	0·017	—	0·022	—	—
5	0·108	0·048	—	0·029	—	—
6	0·124	0·095	—	0·051	—	—
7	0·145	0·123	—	0·069	—	—
8	0·149	0·140	0·012	0·108	0·015	—
9	0·151	0·142	0·084	0·129	0·059	—
10	0·151	0·150	0·112	0·134	0·105	—
11	—	0·155	0·138	0·137	0·128	—
12	—	0·155	0·142	0·138	0·141	0·014
13	—	—	0·144	—	0·141	0·076
14	—	—	0·145	—	—	0·099
15	—	—	—	—	—	0·134
16	—	—	—	—	—	0·150
17	—	—	—	—	—	0·156
18	—	—	—	—	—	0·154

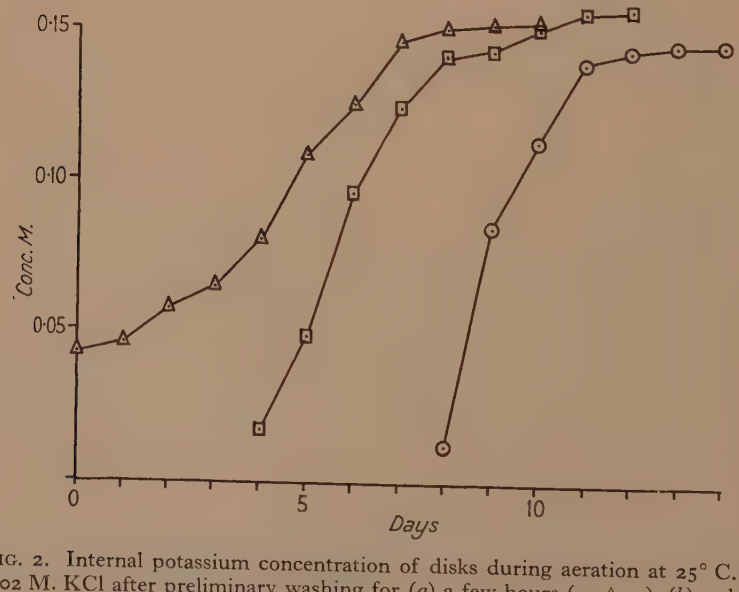

FIG. 2. Internal potassium concentration of disks during aeration at 25° C. in 0·02 M. KCl after preliminary washing for (*a*) a few hours (—△—), (*b*) 4 days (—⊡—), (*c*) 8 days (—⊙—) in distilled water.

disks was about 0·04 M., that is twice the concentration of potassium in the external medium at its maximum value before absorption begins. Subsequent absorption therefore occurs by an accumulatory process, against a concentration gradient throughout the experimental period. Absorption of potassium by disks which had been subjected to a curtailed period of washing of a few hours only took place slowly during the first day after immersion in potassium chloride, but the rate of absorption increased with time and reached a maximum after about 5 days. After this the rate decreased until absorption ceased when the tissue had been immersed for about 8 days and the internal concentration of potassium had reached an estimated value of about 0·15 M. When disks were transferred after cutting to aerated distilled water for more prolonged washing, the internal potassium concentration decreased considerably during the first 24 hours, partly as the result of exudation of salt from the tissue and partly because of the increase in volume. Subsequently the internal concentration decreased more slowly and after 4 days had reached about 0·017 M. When such disks were then placed in potassium chloride solution they absorbed the cation rapidly during the first 24 hours, so that in spite of the initially low value, the potassium concentration had increased after 1 day to a level slightly higher than that attained by freshly cut disks after immersion in salt for the same period of time. During the second interval of 24 hours the rate of absorption of potassium by the washed disks increased to a maximum, and then subsequently decreased until accumulation ceased after about 7 days, when the estimated internal potassium concentration was about the same as that observed for freshly cut disks when absorption was complete. The internal potassium concentration of disks washed in aerated distilled water did not decrease significantly between the 4th and the 8th day of washing, but the rate of absorption of potassium when the tissue was transferred to potassium chloride solution after 8 days' washing was much greater during the first 24 hours than it was when the tissue had been washed for four days. During the 2nd day the rate of accumulation was reduced, and it continued to decrease until absorption stopped after about 4 days. The final potassium concentration attained in these disks was calculated to be 0·144 M. which is not significantly different from the maximum internal concentration observed with the disks washed for shorter periods of time. Other experiments, the results of which are not presented here, showed that a more prolonged washing of the disks for 12 days did not affect either the initial rate of absorption on immersion in potassium chloride, the time during which absorption occurred, or the final concentration of potassium present in the material.

On the other hand, the effect of washing on subsequent absorption of salt appears to be profoundly influenced by temperature during the preliminary period. Table III and Fig. 3 indicate the changes which occurred in the calculated internal potassium concentrations on immersion of disks in potassium chloride solution at 25° C. after washing the tissue for 4, 8, and 12 days in aerated distilled water at room temperature. The data show that after washing at room

temperature for 4 or 8 days disks absorbed potassium during the first 2 hours at a slower rate than did disks washed for the same time at 25° C. but later the rate was greater than that of disks washed at the higher temperature during comparable intervals, so that about the same final internal concentration was obtained, and absorption ceased after approximately the same number of days in each case. The maximum initial rate of absorption was

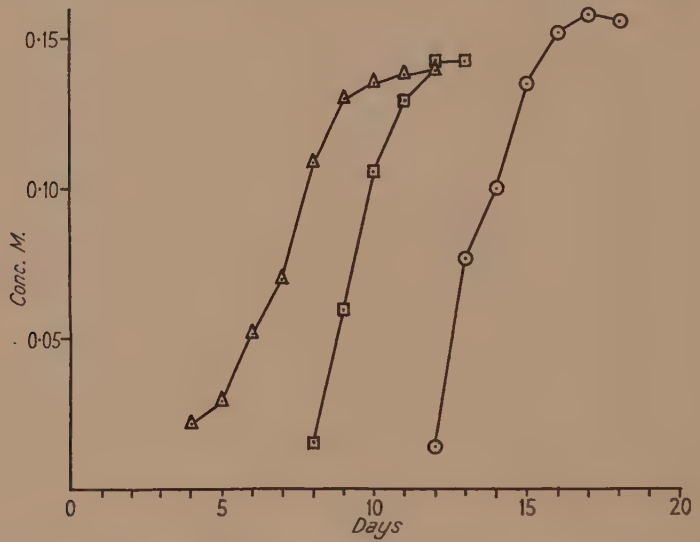

FIG. 3. Internal potassium concentration of disks during aeration at 25° C. in 0·02 M. KCl after preliminary washing for (*a*) 4 days (—△—), (*b*) 8 days (—□—), (*c*) 12 days (—⊙—) in distilled water at room temperature.

developed when the disks were washed for 12 days at room temperature, and in this case the subsequent changes in the rate of accumulation observed were closely similar to those described above for disks which were washed for 8 days at 25° C. prior to the commencement of absorption.

The influence of external salt concentration

Table IV and Fig. 4 indicate the results of an experiment designed to determine the effect of varying external concentration on the accumulation of potassium by disks washed for (*a*) a few hours, (*b*) 8 days, in aerated distilled water at 25° C. before immersion in potassium chloride. Two concentrations of the salt were used, namely, 0·01 M. and 0·04 M. The determinations showed that the internal potassium concentration of disks washed for only a few hours and then transferred to 0·04 M. potassium chloride was not significantly different during the first 2 or 3 days from that of similarly washed tissue placed in 0·01 M. salt solution. During the 4th and 5th days absorption was rather more rapid from the higher external concentration than from the lower, but the total amount of potassium absorbed in the end was not very different in the two treatments. On the other hand, after disks had been washed for

TABLE IV

Internal potassium concentration (M.) of disks at 24-hour intervals during immersion in (A) 0·01 M. KCl, and (B) 0·04 M. KCl, at 25° C., after preliminary washing in aerated distilled water for (a) a few hours, (b) 8 days at the same temperature

Days after cutting	(A)		(B)	
	(*a*)	(*b*)	(*a*)	(*b*)
0	0·035	—	0·035	—
1	0·041	—	0·049	—
2	0·048	—	0·054	—
3	0·056	—	0·068	—
4	0·067	—	0·091	—
5	0·084	—	0·119	—
6	0·102	—	0·130	—
7	0·123	—	0·133	—
8	0·129	0·018	0·138	0·018
9	0·133	0·052	0·139	0·122
10	0·141	0·086	—	0·136
11	—	0·109	—	0·154
12	—	0·119	—	0·156
13	—	0·132	—	—
14	—	0·141	—	—
15	—	0·148	—	—
16	—	0·149	—	—

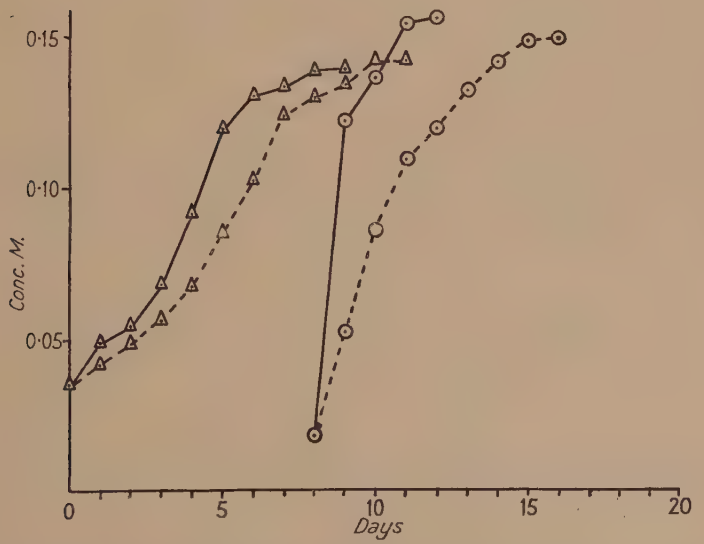

FIG. 4. Internal potassium concentration of disks during aeration in 0·01 M. KCl (broken line) and 0·04 M. KCl (continuous line) after preliminary washing for (*a*) a few hours (△), and (*b*) 8 days (⊙) in distilled water at 25° C.

8 days, the rate of accumulation of potassium from potassium chloride solution at a concentration of 0·04 M. was greater during the first 24 hours than it was

from the salt at the lower concentration. This high initial rate of absorption, however, decreased rapidly, so that even during the 2nd day more potassium was being accumulated from the lower concentration than from the higher one, and this state of affairs continued until absorption stopped, when the total amount of potassium absorbed from each concentration was nearly the same and not significantly different from the amount already observed to be accumulated from 0·02 M. potassium chloride solution.

The influence of potassium cyanide

In view of the sensitivity of salt respiration to cyanide, it seemed necessary to determine the effect of this substance on potassium absorption as well as respiration. The data of Table V and Fig. 5 show that in the presence of 0·001 M. potassium cyanide, freshly cut disks instead of absorbing potassium slowly during the first 24 hours exuded it, and the internal potassium concentration was reduced. Subsequently potassium was absorbed very slowly, and the internal concentration increased towards its initial value, though this was not attained within the experimental period. Disks washed in distilled water at 25° C. for 8 days absorbed potassium at a much reduced rate from potassium chloride solution in the presence of potassium cyanide during the first 24 hours interval, and the rate of accumulation tended to decrease rather with time until the end of the experimental period when the internal concentration attained was about the same as that observed in freshly cut disks after immersion in salt solution with cyanide for an equivalent time.

TABLE V

Internal potassium concentration (M.) of disks at 24-hour intervals during immersion in (A) 0·02 M. KCl, and (B) 0·02 M. KCl 0·001 M. KCN, at 25° C., after preliminary washing in aerated distilled water for (a) a few hours, (b) 8 days, at the same temperature

Days after cutting	(A)		(B)	
	(*a*)	(*b*)	(*a*)	(*b*)
0	0·047	—	0·047	—
1	0·050	—	0·028	—
2	0·058	—	0·026	—
3	0·069	—	0·029	—
4	0·091	—	0·034	—
5	0·105	—	0·032	—
6	0·119	—	0·037	—
7	0·134	—	0·039	—
8	0·139	0·021	0·038	0·021
9	0·142	0·053	0·042	0·028
10	—	0·099	—	0·029
11	—	0·118	—	0·034
12	—	0·140	—	0·032
13	—	0·149	—	0·038
14	—	0·151	—	0·037

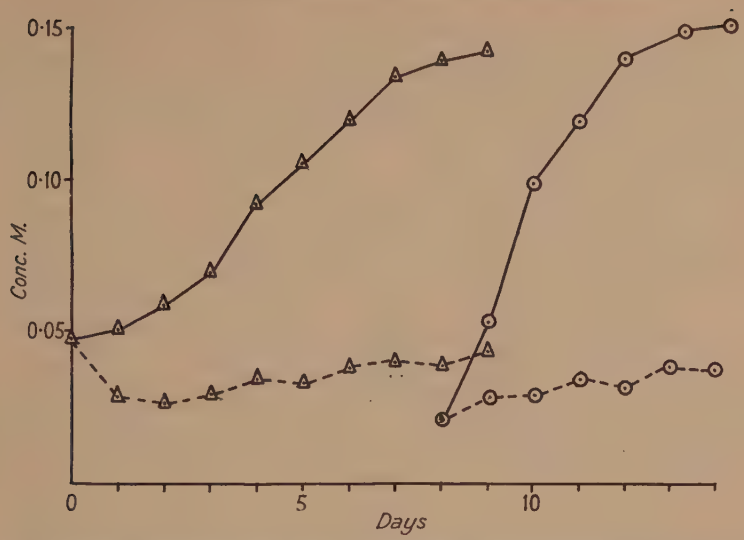

FIG. 5. Internal potassium concentration of disks during aeration in 0·02 M. KCl (continuous lines) and 0·02 M. KCl+0·001 M. KCN (broken lines) after preliminary washing for (*a*) a few hours (△), (*b*) 8 days (⊙) in distilled water at 25° C.

The influence of associated anions

The data of Table VI and Fig. 6 show the effect of different associated anions, chloride, nitrate, bivalent phosphate, and sulphate on the absorption of potassium from solutions of these salts at a concentration of 0·02 M. by disks previously washed for 8 days in aerated distilled water at 25° C. They show that although the general nature of the absorption curves as shown in Fig. 6 is the same in each case, that is, the rate of absorption decreased as internal concentration increased, the actual rates of absorption and the final internal potassium concentration were different with each anion. Accumulation proceeded for about the same length of time from solutions of potassium chloride, nitrate, and bivalent phosphate, but the rate of absorption until accumulation ceased after about 5 days was higher from the solution containing nitrate than from either of the other two, and a correspondingly higher final internal concentration of potassium was recorded. In the same way, absorption was more rapid from the potassium chloride solution than from dipotassium hydrogen phosphate at least until the 4th day, internal potassium concentration remaining higher throughout the experimental period. Potassium was absorbed only relatively slowly from a solution of potassium sulphate, and absorption ceased after about 2 days when the internal potassium concentration was about 0·03 M.

Respiration

The effects of 0·02 M. potassium chloride solution, and also of potassium cyanide at a concentration of 0·001 M. upon the rate of oxygen absorption by

TABLE VI

Internal potassium concentration (M.) of disks at 24-hour intervals during immersion in (A) KCl, (B) KNO_3, (C) K_2HPO_4, (D) K_2SO_4, at a concentration of 0·02 M. after preliminary washing for 8 days in distilled water at 25° C.

Days after cutting	(A)	(B)	(C)	(D)
8	0·014	0·014	0·014	0·014
9	0·061	0·063	0·043	0·025
10	0·095	0·107	0·064	0·029
11	0·116	0·133	0·080	0·029
12	0·132	0·153	0·093	0·032
13	0·138	0·169	0·104	—
14	0·138	0·171	—	—
15	—	0·173	—	—
16	—	0·172	—	—

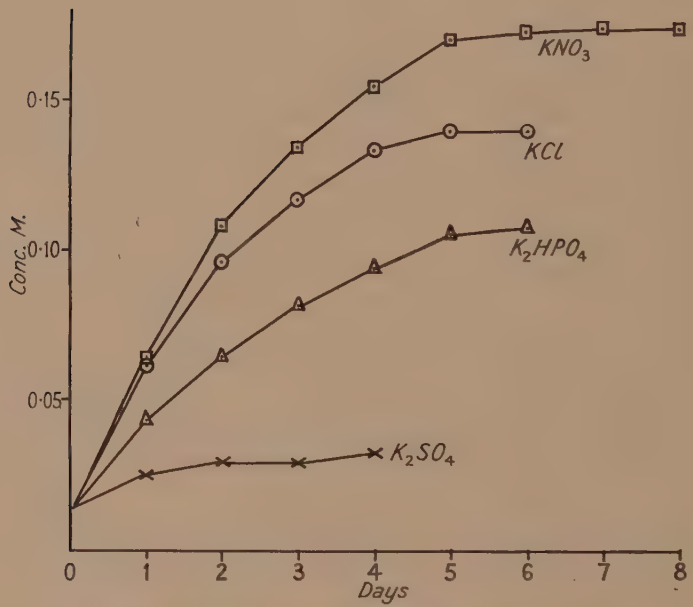

FIG. 6. Internal potassium concentration of disks during aeration at 25° C. in (*a*) KNO_3 (—⊡—), (*b*) KCl (—⊙—), (*c*) K_2HPO_4 (—△—), (*d*) K_2SO_4 (—×—) at a concentration of 0·02 M. after preliminary washing for 8 days in distilled water.

(*a*) freshly cut disks, (*b*) disks washed for a preliminary period of 4 days in aerated distilled water, (*c*) disks washed for 8 days in aerated distilled water before the experimental period, and (*d*) disks washed for 8 days in aerated distilled water, followed by immersion for 5 days in potassium chloride solution before transfer to the Warburg apparatus, were examined, and are represented diagrammatically by Fig. 7–10. Fig. 7 shows that a batch of freshly cut disks placed in distilled water in Warburg flasks absorbed oxygen

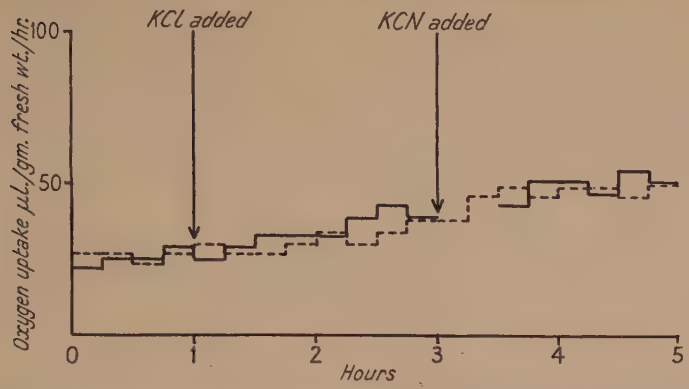

FIG. 7. The effect of 0·02 M. KCl and 0·001 M. KCN on oxygen absorption by freshly cut disks at 25° C. (continuous line). Control in distilled water (broken line).

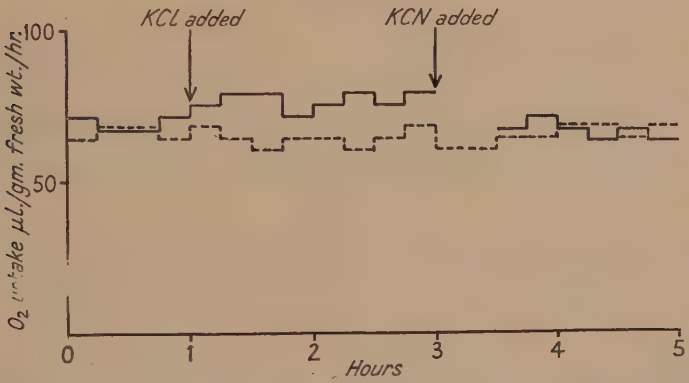

FIG. 8. The effect of 0·02 M. KCl and 0·001 M. KCN on oxygen uptake by disks at 25° C. after preliminary washing in distilled water for 8 days (continuous line). Control in distilled water (broken line).

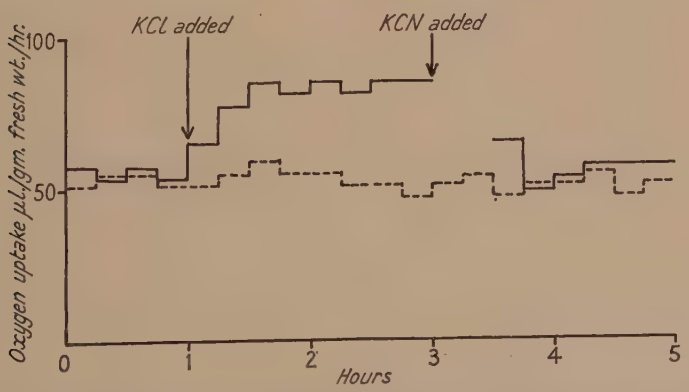

FIG. 9. The effect of 0·02 M. KCl and 0·001 M. KCN on oxygen uptake by disks at 25° C. (continuous line) after preliminary washing for 8 days in distilled water. Control in distilled water (broken line).

during the first hour at the rate of about 25 μl. per g. fresh-weight per hour, and the rate increased steadily during the experimental period to about twice that value after 5 hours, when the experiment was discontinued. The addition of potassium chloride to the medium had no significant effect on the respiration rate, nor was this influenced by potassium cyanide added after 3 hours.

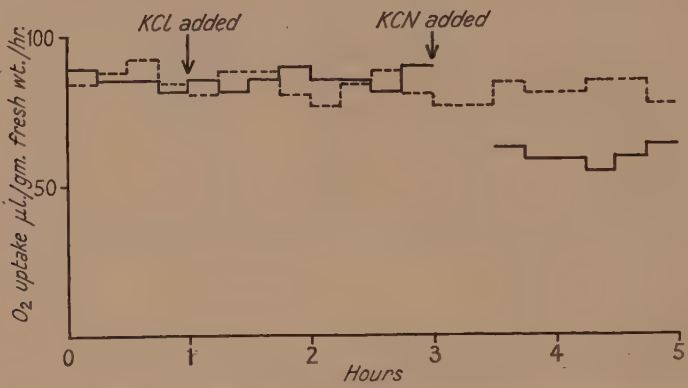

FIG. 10. The effect of 0·02 M. KCl and 0·001 M. KCN on oxygen absorption by disks at 25° C. after preliminary washing in distilled water for 8 days followed by immersion in KCl for 5 days (continous line). Control in distilled water (broken line).

Tissue washed for 4 days and then transferred to distilled water in Warburg flasks absorbed about 65 μl. of oxygen per g. fresh-weight during the first hour, and this rate of respiration remained approximately unchanged during 5 hours. Addition of potassium chloride, however, to the medium in one group of flasks after 1 hour stimulated respiration by about 15 per cent. The rate of oxygen absorption by these disks returned to about its initial value when 0·001 M. potassium cyanide was added (Fig. 8). Fig. 9 indicates that the rate of oxygen absorption by disks washed for 8 days in aerated distilled water was only a little lower initially than that of disks washed for 4 days before respiration determinations were made. But on addition of potassium chloride oxygen absorption was stimulated to about 85 μl./g. fresh-weight/hour, the rate returning again to its initial value in the presence of potassium cyanide. The data of Fig. 10 show that disks which had been washed for 8 days in distilled water and then shaken for 5 days in potassium chloride solution absorbed oxygen at the rate of about 90 μl./g. fresh-weight/hour when placed in distilled water in Warburg flasks. The rate of respiration was unchanged by addition of potassium chloride to the external medium, but oxygen absorption was reduced to about 60 μl./g./hour when potassium cyanide was added at a concentration of 0·001 M.

In a further experiment, the results of which are indicated by Fig. 11, the effect of potassium salts containing anions other than chloride, namely, nitrate, bivalent phosphate, and sulphate, on respiration of disks washed for a preliminary period of 8 days in aerated distilled water was examined. It was

observed that the rate of oxygen absorption was stimulated by each of these salts at a concentration of 0·02 M. to the same extent as by potassium chloride, and this additional respiration was again sensitive to potassium cyanide poisoning.

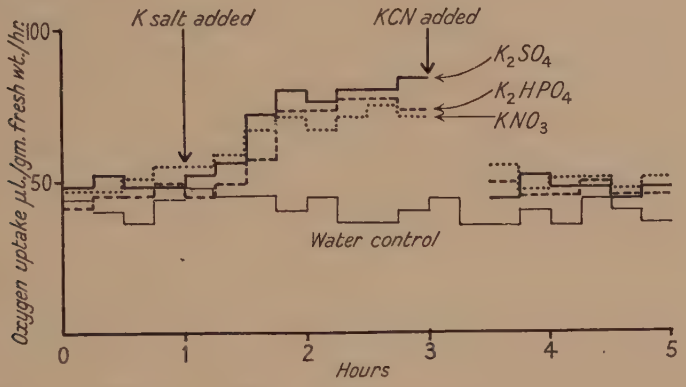

FIG. 11. The effect of 0·02 M. KNO_3 (dotted line), 0·02 M. K_2HPO_4 (broken line), 0·02 M. K_2SO_4 (thick line), and KCN on oxygen uptake by disks after preliminary washing for 8 days in distilled water. Control in distilled water (thin continuous line).

DISCUSSION

It is clear from the data presented above in Table III and Fig. 3 that the relationship between potassium absorption and the internal concentration of this ion is not always a simple one. Freshly cut disks, for example, absorb salt relatively slowly at first, and then more rapidly. The internal concentration of potassium thus increases, but the rate finally decreases again as the maximum internal concentration is approached. A similar situation exists in relation to disks which have been washed for 4 days in water before placing in salt solution, though here the initial period during which the rate of absorption increases is not so prolonged. The slow rate of salt absorption by freshly cut disks of storage tissue immediately after immersion in nutrient solutions frequently observed by other workers has received in the past various explanations. Steward and Preston (1940), for example, have suggested that increasing absorption of mineral salt ions by potato disks is associated with increasing metabolic activity of the actively dividing cells on the surface of the disks, and in particular with protein synthesis. Although no protein determinations have yet been made in the present investigation with beet tissue, no callus formation has been observed, and it would appear here that the growth of cells either by division and expansion, or by expansion alone, is not essential for the development of the capacity to absorb ions rapidly. Nor does it seem likely that the high initial salt-content of the disks is an important factor as Stiles and Skelding (1940) postulated. There is no significant difference in the potassium content of disks after washing for 4 days in distilled water at 25° C. and at room temperature, yet the absorption of potassium on subsequent

immersion in potassium chloride solution is considerably greater for several days in the former case than in the latter (see Figs. 2 and 3). Rees and Skelding (1950) have recently claimed to show that there is present in beet root tissue a substance which inhibits salt absorption and disappears during washing of the material, so accounting for the development of rapid salt absorption. So far it has not been possible for us to confirm this suggestion in the present experiments, though this has been attempted. On the other hand, it seems evident that the development of maximum absorptive capacity is related to some aerobic chemical process or processes, since it is affected by temperature during the preliminary washing, and also by aeration and respiratory poisons. Moreover, it appears to be associated with the development of a respiratory component which is characterized by being unusually sensitive to cyanide and only appears in the presence of transportable ions. In these respects it agrees with the 'salt respiration' of Robertson and his collaborators, and these observations appear to support the Lundegårdh hypothesis that there is a close connexion between salt absorption and this particular component of respiration.

Once the maximum absorptive capacity of the tissue has been established, the results presented in Tables III and IV and Figs. 2, 3, and 4 show that there is a close relationship between potassium absorption and the internal concentration of this ion. The rate of absorption of potassium by disks which have been washed in distilled water for a preliminary period of 8 days at 25° C., or 12 days at room temperature, decreases continuously as internal concentration increases until accumulation ceases. The relationship is clearly shown by Fig. 4, which indicates that though the rate of absorption is more rapid at first from a higher concentration of salt, the rate decreases more quickly, as the maximum internal concentration of potassium is attained, and the final internal concentration established is in each case about the same. The establishment of such a connexion between internal concentration and the rate of absorption of potassium does not necessarily indicate that the relationship between the two is a very direct one since the absorption and internal concentration of the associated anion must also be considered. It is well known that the absorption of a particular ion from a mineral salt solution is profoundly influenced by the presence of other ions in the medium, and the effect of different anions on the absorption of potassium is indicated above in Table VI and Fig. 6. Determinations of chloride ion absorption from potassium chloride solutions by beet root disks have shown that for each 24-hour interval until absorption is complete the ratio of potassium absorption/chloride absorption does not differ significantly from one. It is possible therefore that the accumulation of potassium is limited by the rate of absorption of the anion, and that uptake of potassium may cease when the internal concentration of the anion has reached a limiting value, so that absorption of anions cease. Further research is required to elucidate this point.

Whether the high internal concentration of cations, or anions, or both is responsible, it is evident from the data presented above that in well-washed

tissue the rate of accumulation of potassium decreases as the internal mineral salt-content increases. If there exists the direct relationship between ion accumulation and 'salt respiration' that Lundegårdh postulated, it would be expected that as the rate of absorption of cations and anions decreased there would also be a reduction in the cyanide sensitive component of respiration. In fact the data of Fig. 11 show that even after salt absorption has ceased the magnitude of 'salt respiration' remains the same as it was when accumulation was proceeding rapidly. This apparent anomaly may be explained without discarding the Lundegårdh hypothesis if it is assumed that the cell protoplasts are not completely impermeable to the outward diffusion of ions from the vacuoles into the external medium. In that case the rate of leakage will increase with increasing internal concentration, and the point at which apparent absorption ceases may be that at which accumulation proceeding at a uniform rate indicated by the level of 'salt respiration' is balanced by outward diffusion. This possibility is easily examined since if the protoplasts are appreciably permeable to outward diffusion of ions it ought to be possible to detect an increase in the external concentration of potassium, for example, when tissue containing a high internal potassium concentration is placed in a medium containing a low concentration of cyanide which effectively prevents absorption. No significant leakage of potassium from the tissue under these conditions has been observed with beet root disks, so that some other explanation than the above is necessary to account for the observed facts. It is clear that as internal concentration of salt increases the ratio of salt accumulation to salt respiration decreases and attains zero when accumulation ceases. This may mean that more respiratory energy is required for the absorption of a given number of ions as the internal concentration becomes greater either because more work is being done to overcome the increased concentration gradient or for some other reason. Jacques (1938) has suggested that increasing internal osmotic pressure in cells of *Valonia macrophysa* may reduce the rate of accumulation of ions by causing the protoplast to become dehydrated, and thus present a greater resistance to movement of ions.

Whatever the explanation of the influence of internal salt concentration, it seems unlikely from the evidence available that the relationship between accumulation and salt respiration can be as intimate as the Lundegårdh hypothesis suggests, at least with the material and under the conditions of the experiments described above. Not only does the level of salt respiration remain apparently the same when potassium and chloride ions are being absorbed at different rates during the course of an experiment, but the data of Fig. 11 show that respiration is stimulated to a comparable extent by three different potassium salt solutions containing potassium at the same molecular concentration, although Fig. 6 indicates that in each case potassium is being absorbed at a different rate.

From their investigation of the influence of external salt concentration on salt accumulation and respiration, Robertson and Wilkins (1948) were led to the conclusion that stimulation of respiration in the presence of mineral salt

ions is not the result of ion transportation, but may perhaps be due to some other effect of the ions, whether moving or not, on the respiratory system. This hypothesis is supported by the results which are presented here.

ACKNOWLEDGEMENTS

We wish to thank Professor T. A. Bennet-Clark for the benefit of many stimulating discussions during the course of this research, and also members of the laboratory staff of the Department of Botany, King's College, London, for technical assistance.

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Physiological Studies in the Mucorales

PART I

THE PHOTOTROPISM OF SPORANGIOPHORES OF *PHYCOMYCES BLAKESLEEANUS*

G. H. BANBURY[1]

(*Received 31 July 1951*)

SUMMARY

Previous views on the physical basis of phototropism in *Phycomyces* sporangiophores are briefly discussed.

(i) It was confirmed that unilaterally illuminated sporangiophores immersed in liquid paraffin show strong negative phototropism.

(ii) Elongation growth ceased and no phototropic response took place under anaerobic conditions.

(iii) By focusing a fine beam of light on to one edge of the growing zone of a sporangiophore, leaving the other side in darkness, it was established that greater elongation took place in the illuminated zone, the sporangiophore tending to bend out of the beam. Rapid reversal of the curvature followed when the illumination was transferred to the opposite edge of the sporangiophore.

Wassink and Boumann's suggestion that phototropism can be initiated by a one-quantum-per-cell process is criticized in the light of this result and other work by Castle.

INTRODUCTION

THE phototropic behaviour of *Phycomyces* was first extensively studied by Blaauw (1909, 1914, 1915, 1918). He showed that, within the light-intensity range of normal response, illumination stimulated the growth of the sporangiophore, and that the sensitive zone was in the apical growing region.

Blaauw interpreted the phototropic curvature as a consequence of the unequal positive light-growth response of the near and far sides of the unilaterally illuminated sporangiophore. He stated that since the sporangiophore was physically a cylindrical lens, the far side was more strongly illuminated than the near, and so grew more rapidly. He and Nuernbergk (1927) assumed that the small, brightly illuminated zone had a preponderant effect on growth distribution.

Now, as Castle (1933) pointed out, this would imply that a certain amount of light energy if concentrated in a small volume of cell would be more effective than if spread over a larger volume, but, as Blaauw himself also showed, within wide limits the mass photo-response is proportional to the product—(intensity of illumination × duration of exposure), i.e. to total light energy absorbed.

Therefore this explanation would only be adequate if there were minute locally differentiated zones of reception, rather widely spaced, within the cell.

[1] The bulk of the work described here was carried out in the Botany Department of King's College, University of London, during 1948–9.

These have not been generally recognized in *Phycomyces*, though one do exist at the base of the more specialized subsporangial swelling of *Pilobol* However, Heuckel (1927) claimed that in *Mucor mucedo* as well as in *Pilobol crystallinus* the protoplasm was demonstrably denser against the wall on th far side of the unilaterally illuminated sporangiophore, where growth is mo rapid. This paper, a communication from the State University of Perm, th writer only discovered by fortunate chance, and it seems to have been misse by other workers in this field. Clearly if Heuckel is correct in his interpretatic it is of great importance to the understanding of the phototropism of sing cells, but apparently he only observed this uneven distribution of opacity aft fixing the sporangiophores with boiling corrosive sublimate, which might we cause contraction of cytoplasm and varying opacity.

Oehlkers (1926), considering lens action alone inadequate, suggested th total internal reflection at the back wall of rays passing through the peripher parts of the cell would extend the effective absorptive path of light within th far half of the cell.

Unfortunately, as Castle observed, 'the total internal reflection diagramme in Oehlker's paper is physically impossible, as a glance at his incorrect ge metrical construction will show'. There is, of course, some, but at no poi total, internal reflection of light from the back wall of the sporangiophore.

Castle has carried out a long series of elegant and precise studies of th growth and phototropism of these sporangiophores (1930, 1931, 1932, 193 1934). In one of the papers already cited (1933) he gave the result of h experimental determination of the refractive index of the contents of th sporangiophore as 1·38. Then, having assumed that the action of light primarily on uniform cytoplasm, he showed in a comprehensive mathematic consideration of the case of a unilaterally illuminated sporangiophore that tw important conclusions can be drawn: (i) The mean length of path of light i the back half of the sporangiophore is 1·26 times that in the front hal (ii) Allowing for reflection, more energy is absorbed in the far half than in th front, provided that the cell contents' absorption coefficient (α) has a valu not greater than 4 for large and 8 for small sporangiophores. So far α has n been determined experimentally.

Reference should be made to Castle's papers for details; he sums up hi thesis in the sentence: 'The longer total path of light rays through the proto plasm of the far half of the cells is the significant factor in the phototropism o relatively transparent cells.'

It is often assumed that the fact that light is concentrated in the back ha of the sporangiophore is in itself sufficient to explain the larger growth respons there. This could only be the case if the protoplasm were anomalously sensi tive to higher light intensities. Apart from experimental observations o phototropism and light-growth reactions over a wide range of light intensities and photochemical considerations, all of which cast doubt on such an assump tion, it is inconsistent with the results of the ingenious experiments of Bude (1918). Buder found that sporangiophores immersed in liquid paraffin o

refractive index 1·47 showed strong negative phototropism, curving away from the light source. There was thus a differential acceleration of growth without concentration of light to a high intensity on the near side.

The experiments now to be described were designed to eliminate a few remaining uncertainties in the work summarized above. In particular it seemed desirable to devise an unambiguous test of the unproven assumption that when more light energy is absorbed in one side of a sporangiophore than in the other, then the greater growth rate will be found on the side which is absorbing more energy, so producing a curvature convex on that side.

EXPERIMENTAL PROCEDURES

Various (+) and (−) strains of *Phycomyces blakesleeanus* Burgeff were used in this work. They differed somewhat among themselves in the mean size and rate of growth of their sporangiophores, but there were no significant qualitative differences in their behaviour in these tests.

For the experiments the moulds were grown at room temperature on 2 per cent. malt extract agar: stocks were maintained on malt agar and on autoclaved bread paste.

Variations in opacity

It may first be mentioned that repeated attempts were made to confirm the observations described by Heuckel (1927), but using living sporangiophores. No such uneven opacity was detected in the growing regions of sporangiophores during or immediately after unilateral illumination. Complete failure has to be reported after careful microscopic observation of phototropically responding sporangiophores of *Mucur mucedo* and also with those of *Phycomyces* which are similar but larger and easier to study. If any such differences existed they were undetected by eye despite the successive use of various systems of illumination by converging, parallel, and diverging beams incident obliquely on or transmitted directly through the sporangiophores. The differences in opacity arising as a photo-response must indeed be small if they exist at all.

It seems reasonable to suppose that such local changes in the absorption coefficient, if they do occur, will, at least over a limitedrange of light intensities, vary in a manner directly related to the light energy absorbed in the region in question. Then if a sporangiophore were transferred from darkness to unilateral illumination the initial effect would be some increase in opacity throughout the cytoplasm of the responding zone, and with the greatest increase in the far half. From Castle's calculations it will be evident that any general increase in α reduces the value of the fraction

$$\frac{\text{Total light energy absorbed in the rear half}}{\text{Total light energy absorbed in the front half,}}$$

so that on *a priori* grounds the interpretation of Heuckel seems improbable.

ii. *The phototropic reversal in paraffin*

Repeating Buder's experiment with sporangiophores grown in the dark a then submerged in liquid paraffin and unilaterally illuminated, it was fou that the sporangiophores bent over very sharply away from the sou through 90° or rather more and then straightened out and continued to gr along the path of the beam. The possibility that this result might be associa with the relatively anaerobic conditions under which the response occur was eliminated by repeating with sporangiophores growing in vessels fr which some or all of the atmospheric oxygen was displaced by nitrogen. negative phototropic curvatures occurred: instead growth was checked

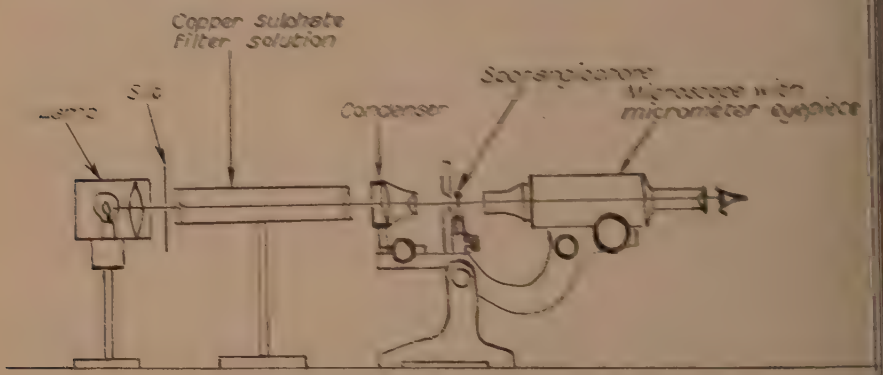


FIG. 1. Apparatus as assembled (see text).

only a slight positive curvature could be detected. No growth or curvat could be detected when the last trace of oxygen was removed with alkali pyrogallol, though pronounced 'guttation' took place. It can be conclud that Buder's interpretation of his experimental results was correct.

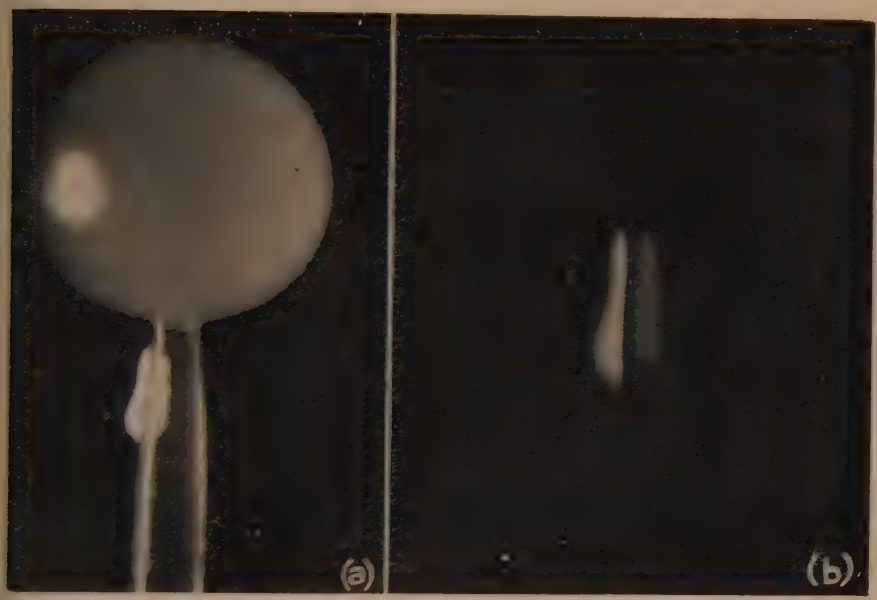
iii. *Unilateral light absorption*

It was clearly desirable to determine what would happen in the critical c of a sporangiophore illuminated along one edge in such a way that a narr vertical column of cytoplasm on one side was absorbing light energy while t rest of the cell remained in darkness. Such a sporangiophore would be expect on current theory to grow more rapidly on the illuminated side.

Preliminary attempts to secure the desired illumination by using po sources produced by drawing out glass rods to a fine tip and using them light conduits, or by shading the greater part of a sporangiophore with knife-edge, gave little promise of success, but the arrangement of apparat now to be described proved quite simple and convenient in use. It might a be applicable to other related studies.

Fig. 1 illustrates the complete assembly, which was carefully alined on firm bench. The observations were made in a fully darkened room at tim when vibration was least troublesome.

Light from the lamp filament passing through a slit aperture about 1 mm. / ·3 mm. was filtered through a 20-cm. trough of 2·5 per cent. $CuSO_4$ solution o absorb infra-red rays. (The need for such a precaution was demonstrated by Vortman as early as 1883.) The band of light was then focused on to one side f the growing sporangiophore by the condenser lenses behind an iris dia- hram stopped down to 2 mm. This was then observed through a microscope rovided with a micrometer eyepiece.



(*a*) (*b*)

FIG. 2. Photograph of Sporangiophore.

(*a*) With supplementary illumination, (*b*) as it appeared during the experiment. The spot of light was approximately 100 μ / 30 μ.

The sporangiophore was growing on agar in a slide cell mounted on the echanical stage and protected by cardboard draught shields. Whether these creens were moisture saturated or left dry throughout appeared to make no ifference to the behaviour of the sporangiophores as studied. However, in ew of the appreciable negative hydrotropism attributed to *Phycomyces* porangiophores by, for example, Walter (1921), dry screens were even- ually used.

The appearance with and without supplementary illumination of the porangiophore as viewed through the measuring eyepiece can be seen in the notographs (Fig. 2, *a* and *b*). The sporangiophore was illuminated along one dge by the narrow band of light. The more rapid growth of the illuminated de caused the sporangiophore to tend to curve out of the beam which razed' it, and intermittent adjustment of the mechanical stage maintained

the sporangiophore in such a position that its edge just intersected the imag of the slit.

The horizontal displacement of the edge of the sporangiophore was measure by noting the micrometer eyepiece readings at definite successive time inter vals (usually of half or one minute), and periodically the rate of vertica elongation was also noted, using the same micrometer eyepiece rotated throug 90°. The vertical growth rate of the sporangiophores under these experimenta conditions was rather slow, but nearly constant over the observation period.

The actual movement of the sporangiophore could be calculated from th readings taken after corrections had been made for the bodily displacement o the sporangiophore when the mechanical stage was moved. Corrections wer simply based on the rate and direction of growth and the actual readings immediately before and after adjustment of position, and the time taken fo that adjustment, which was usually one minute. Horizontal displacement wa then plotted against time elapsed.

Two typical sets of results obtained by this method are set out in Fig. 3 which should be self-explanatory.

The sporangiophore with a terminal sporangium chanced to elongate a a rate of 10 μ a minute, so the graph as drawn also represents the movement in space of a point at the base of the sporangium.

It may be noted that when the curvature had continued for a relatively lon duration in one direction, a longer 'reaction' period elapsed before the reversa of the illuminations was followed by a reversal of curvature. One hopes that i may be possible subsequently to undertake a more critical study of the latenc and reaction times of sporangiophores of various dimensions using a modifica tion of this technique, and so to develop the stimulating suggestions of Castl and Honeyman (1934). With a higher growth rate and more intense illumina tion more precise observations may be possible.

DISCUSSION

The results presented above are, it is considered, fully in accordance wit Castle's theoretical deductions and show clearly that growth of the sporangio phore is most rapid in the region in which most light energy is absorbed. I the unilaterally illuminated sporangiophore growing in air this region is in th rear half of the hypha, not that nearer to the source.

An entirely different approach to the problem is to be found in a paper b Wassink and Bouman (1947).

By analysing data obtained by Blaauw in 1909 they have attempted t determine the minimum number of light quanta essential for initiation of phototropic curvature in *Phycomyces* and *Avena*. Plotting Blaauw's data for th percentage of sporangiophores curved against the log of incident light energ an S-shaped curve is obtained, the steepness of which should be an indicatio of the number of quanta required to produce the reaction.

Blaauw's results for *Phycomyces* fit best to a one-quantum curve; for *Aven* a much higher number would appear to be required.

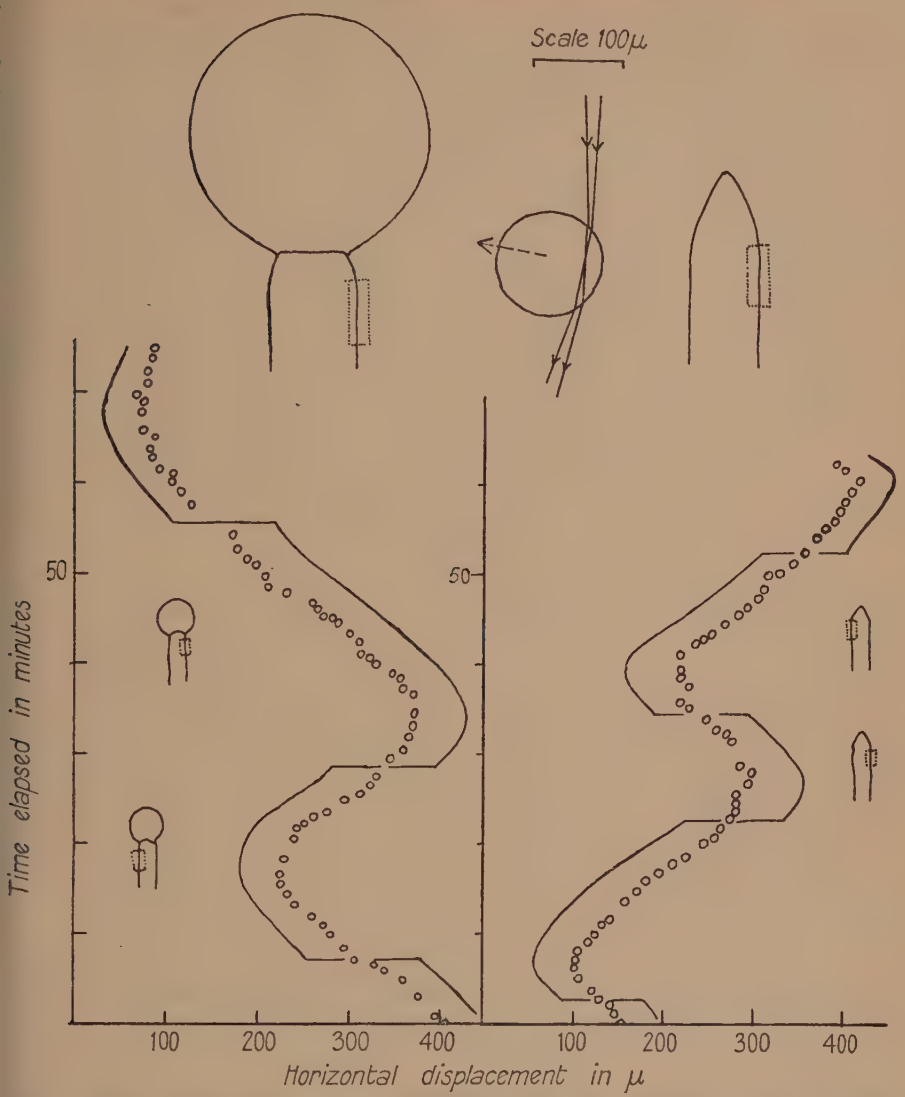


FIG. 3. Upper portion: scale drawings indicating sizes and illuminated regions of sporangiophores used. Lower portion: successive positions (o o o o) of fixed point on a sporangiophore. The lighted side is indicated thus (——), the darker side (o o o). Sporangiophore with sporangium on left; young sporangiophore on right.

Again, plotting log per cent. of sporangiophores not showing a curvature response against a linear scale of incident light energy, these results appear to fit the shallow sloping line for a one-quantum per cell process better than the steeper curves for two or more quanta.

The writer is not competent critically to judge the validity of this quantum-statistical reasoning, or to say whether the occurrence of some other uni-

molecular reaction limiting the response could give a similar result without the need for postulating that a photon effectively absorbed will lead to a curvature towards the source of emission. One implication of the interpretation advanced by Wassink and Bouman is, however, quite clear. They take the view that one photon effectively absorbed leads to a measurable curvature, and they suggest that degrees of curvature corresponding with 1, 2, 3, or 4 photons absorbed in a limited cell volume and time might be exhibited. It is, as they suggest, desirable that this hypothesis should be tested experimentally, though the inherent practical difficulties will be formidable. In the meantime it is of

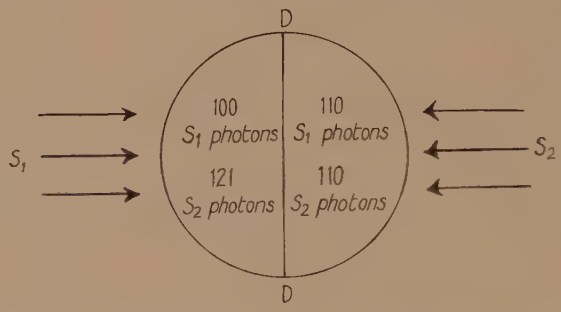


FIG. 4. T. S. Sporangiophore. Bilaterally illuminated (see text).

interest to point out the serious objections to their interpretation which arise from Castle's theoretical treatment and the results of the experiments described above which appear to show that the region in which the photon is absorbed must be important. As Professor Bennet-Clark, who first drew the writer's attention to this paper, has cogently argued, photons effectively absorbed near the front surface would be expected to tend to make the *Phycomyces* curve *away* from a unilateral source, those absorbed near the back *towards* it, and those in the middle merely to produce a stimulus to vertical elongation.

It is difficult to envisage any process by which a single photon when effectively absorbed could stimulate a tropic response directly related in direction to the external path it had previously followed. On the other hand, when large numbers of photons are considered one can speak in terms of the statistical distribution of photon absorption throughout the hypha; thus with unilateral illumination it is rather more probable that a photon entering the sporangiophore will be absorbed in the rear half than in the front, and for a specific case actual probability values could be calculated.

It seems clear that quite a small excess of light energy continually absorbed in one half of a sporangiophore will result in a greater growth rate on that side and consequent curvature despite the uniform gravitational stimulus to which the sporangiophore is also exposed. It is well known (Castle, 1933) that a *Phycomyces* sporangiophore when illuminated from two opposite sides can distinguish between the two light fluxes and bend towards the stronger source when they differ by 10 per cent.

Suppose that the values α and DD' (Fig. 4) for this sporangiophore are such that on unilateral illumination the value of the fraction

$$\frac{\text{Total light energy absorbed in front half}}{\text{Total light energy absorbed in rear half}} \text{ is } \frac{1}{1{\cdot}1}.$$

Consider two equidistant and opposed sources S_1 and S_2 such that

$$S_2 = \frac{110}{100} S_1.$$

If the rate of photon absorption per unit time with source S_1 alone is 100 in the side facing S_1 and 110 in the rear and with source S_2 alone is 110 in the side facing S_2 and 121 in the rear, then a sporangiophore with the properties we have assumed will show a curvature towards the stronger source S_2, there being a steady difference between the energy absorbed in the opposite halves of only 1/441, or 0·23 per cent. of the total light energy absorbed.

Even if a value of 1/1·2 is taken for the fraction

$$\frac{\text{Total light absorbed in front half}}{\text{Total light absorbed in rear half}}$$

the energy difference under the above conditions will be less than 0·5 per cent. of the total energy absorbed, implying a remarkably fine discriminatory mechanism.

Physiological Studies in the Mucorales

PART II

SOME OBSERVATIONS ON GROWTH REGULATION IN THE SPORANGIOPHORE OF *PHYCOMYCES*

G. H. BANBURY

(Received 31 July 1951)

SUMMARY

Lanolin emulsion paste incorporating indolylacetic acid in concentrations of 10,000, 1,000, 100, and 10 μg./ml. when applied with a micromanipulator to one side of *Phycomyces* sporangiophores had no clearly marked action on subsequent growth.

Griseofulvin, a metabolic product of *Penicillium janczewskii* and certain other moulds, at a concentration of 100 μg./ml., produced a local increase in the rate of extension of the wall at or near the region to which it was similarly applied, with consequent curvature from the normal erect growth habit.

Previously proposed explanations of the regulation of sporangiophore growth, and of phototropism, in terms of the action of heteroauxin and auxin-*a* are criticized, and attention directed to the danger of assuming that substances active in regulating the growth of cellulose-walled tissues will have a similar action on chitin-walled fungal hyphae.

INTRODUCTION

THE pioneer work on 'growth substances' in mould mycelium and metabolite fluid was necessarily confused, since several physiologically active substances were often present together in the solutions tested. The earlier publications dealing with 'auxins' need not be reviewed in detail here since they are fully discussed in papers by Nielsen (1930), Ronsdorf (1935), and Thimann (1935).

Ronsdorf (1935) quotes Kögl as having identified heteroauxin as a constituent of the 'Rhizopin' produced by *Rhizopus suinus* which was studied by Nielsen (1930) and by Thimann (1935). Heyn (1935) identified indolylacetic acid in ether extracts of *Phycomyces* by agar diffusion rate measurements.

Ronsdorf herself found that 'auxin-*a*' had no action on the weight of the final yield of *Aspergillus niger* and *Ophiobolus graminis* cultures.

Leonian and Lilly (1937) found that heteroauxin in concentrations of 1 in 10^4 to 1 in 10^7 did not under any of the conditions tested promote the growth of any of the wide range of zygomycetous fungi tested. Neither vitamin-requiring species nor those growing in simple synthetic media showed any enhanced growth rate or yield, nor was heteroauxin plus yeast extract more beneficial to the exacting strains than yeast alone.

Various members of the Saprolegniaceae, which have cellulose hyphal walls, were found by Murdia (1939) to be unaffected by 1 in 10^6 and 1 in 10^7 of heteroauxin, while higher concentrations were inhibiting.

Janke and Sorgo (1939) found that heteroauxin had no action on yeast,

Aspergillus, and *Rhizopus* cultures, and on the yield of *Phycomyces* 'a slight co-growth-substance action with thiamin'.

The experiments of all these workers were designed to detect any general vitamin-like growth promoting action by heteroauxin rather than to investigate whether the 'auxins' active on higher green plants play any part in regulating the pattern of growth in fungi or in directional tropic responses to stimuli.

The first attempt at an explanation of the mechanism of the light-growth response and phototropism in *Phycomyces* was made by van Overbeek (1939). First he pointed out that since the growth substance detected in *Phycomyces* was heteroauxin, the possibility of photo-inactivation would not be involved. That this is not necessarily the case has since been shown by Galston (1949) and Galston and Baker (1949), who described riboflavin sensitized photo-oxidation of indoleacetic acid and various enzyme systems, and by Ferri (1951), who suggests that the destruction of indoleacetic acid may be connected with the presence of a fluorescent substance in the solution. Van Overbeek then pointed out that the work of Bonner (1934) and van Santen (1938) had shown that increased acidity in a medium tended to result in increased auxin activity through reduction of dissociation, and that Brauner and Brauner (1938) had demonstrated that light changes the permeability of certain membranes, favouring penetration by negative ions over positive. He therefore tentatively postulated that illumination might, by affecting the relative mobilities of H^+ and OH^- ions, increase the acidity and in consequence the heteroauxin activity at the growing surface of the sporangiophore. This possible explanation he put forward with the suggestion that it might indicate the lines along which further investigations could profitably be developed; the experiments to be described were commenced with this in mind.

Kögl and Verkaaik (1944) reported the presence of both indolylacetic acid and auxin-*a* in *Phycomyces* sporangiophores. The mould was grown and harvested, and the acid and ether extract prepared and vacuum dried, in the dark or in phototropically inactive red light. They found that about 96 per cent. of the extracted auxin activity (measured by the *Avena* coleoptile technique) was destroyed by hot 5 per cent. hydrochloric acid, and that by the diffusion test that activity was associated with a molecular weight of about 175, corresponding with heteroauxin. The remaining 4 per cent., acid-resistant but destroyed by alkali, gave a diffusion test molecular weight value of about 330, so agreeing with the properties attributed to auxin-*a*.

Kögl and Verkaaik therefore concluded that the phototropism of *Phycomyces* was to be attributed to the inactivation of auxin-*a* or some closely related substance in the presence of β-carotene. The acid-resistant auxin fraction from *Phycomyces* was, they state, found by Koningsberger and Verkaaik to regenerate phototropic sensitivity in twice decapitated *Avena* coleoptiles, and was thus physiologically equivalent to auxin-*a*. This and related work is briefly discussed by Galston (1950).

Brian (1946, 1949) has shown that griseofulvin or 'curling factor', a metabolic product of *Penicillium janczewskii* and *P. griseofulvum*, when included in culture media in concentrations as low as 0·1 μg./ml., caused chitin-walled, but not cellulose-walled, fungal hyphae to grow in spiral form; higher concentrations, 1–10 μg./ml., resulted in uncoordinated irregular swellings of the hyphae. He suggests that 'griseofulvin is either itself a growth regulating substance for chitin-walled fungi or it specifically interferes with a growth regulating system in such fungi'.

It seemed desirable that a test should be made of whether heteroauxin unilaterally applied to the outside surface of the growing region of a sporangiophore would result in a tropic response. During a demonstration by the writer in 1949 of the earlier experiments on these lines, reference was made by Dr. P. W. Brian to the possible significance in this connexion of griseofulvin. The writer is very grateful to Dr. Brian for the information he provided and for a most generous gift of a sample of this substance for test in the same manner as used for heteroauxin.

EXPERIMENTAL PROCEDURES

The indolylacetic acid and the griseofulvin dilutions were incorporated in lanoline-water-paraffin pastes. After a few tests with difference proportions the ratio 6 : 3 : 1 by volume at about 65° C. was adopted. The ingredients were measured out with a 20-ml. glass syringe fitted with a coarse serum needle, the appropriate dilution of the growth substance in a small volume of ethanol added with a precision syringe, and the emulsion kept hot and stirred for about 15 minutes to drive off the alcohol. It was then allowed to cool while being stirred, and a smooth creamy yellow paste resulted.

Pastes containing 10,000, 1,000, 100, and 10 μg./ml. of heteroauxin and 100 μg./ml. of griseofulvin were prepared. The 10 μg./ml. heteroauxin paste was qualitatively checked for activity by unilateral application to *Avena* coleoptiles and found to be satisfactory: the griseofulvin paste and plain paste had no effect on the coleoptiles.

Slide culture troughs were constructed by cementing glass slides with Durofix on to either side of spacing-pieces. These were shaped from 3 mm. diameter glass rods 11 cm. long by bending at each end through 90° in the same plane, and then grinding the sides flat to ensure a good fit against the glass slides. These troughs withstand mild dry sterilization at 110° C. for 20 minutes, which is sufficient for the work described. They will stand upright in a slide box and will fit conveniently on to the mechanical stage of a microscope.

Two per cent. malt extract agar was poured into the slide culture troughs, and they were inoculated at one end with a fragment of young mycelium of *Phycomyces blakesleeanus*, and placed upright in complete darkness at room temperature. All subsequent operations were carried out by red 'photographic' safe-light. (Sporangiophores when tested close to this source showed no phototropic response after 12 hours' unilateral illumination.) When the

sporangiophores had sufficiently developed, all but the most robust were carefully plucked out, and each slide trough in turn was then placed in position vertically on the stage of a horizontally inclined microscope. A Chambers micromanipulator had previously been clamped to the side of the stage in such a position that it was conveniently possible lightly to touch points on one side of the erecting sporangiophores with a fine needle. After largely unsuccessful preliminary attempts to construct a microsyringe capable of extruding

FIG. 1.

minutely regulated quantities of the prepared paste, the simple expedient was adopted of dipping the tip of a solid glass needle into the paste and then withdrawing it. The tip was then found to be coated with a sheath of paste drawn out somewhat beyond the glass. The micromanipulator was employed to touch this paste sheath against a point on the wall of the sporangiophore just below the apex. Gentle retraction often then left a small patch of paste at the point touched (see Fig. 1). Measurement of its dimensions and comparison with models made of plasticine applied to a length of 2-cm. bore glass tubing gave when required an approximate estimate of the dose applied.

The treated sporangiophores were left to continue growth in the dark for periods varying from 20 to 120 minutes and were then observed. The degree and direction of all curvatures in the sporangiophores were noted, and in some instances they were photographed.

A similar routine was followed for all trials, whether heteroauxin paste griseofulvin paste, or plain paste was employed.

RESULTS AND DISCUSSION

i. *General considerations*

In assessing work of the kind described it is important to remember that *Phycomyces* sporangiophores are sensitive to touch stimulation, or, at least they sometimes grow abnormally after a limited amount of prodding in the growing region with a micromanipulator needle. This occasionally occurred despite the fact that no initially visible injury had thus been produced. Such effects were observed and discussed by Graser (1919) and Kirchheimer (1933). Sometimes the sporangiophore bends in the region of application of the stimulus, or a branch sporangiophore develops there, and the growth of the 'main apex' may then be arrested. It is suggestive that atypical growth is also sometimes observed when sporangiophores are transferred to a humid atmosphere and their substrate is diluted with water, so presumably producing a high internal turgor pressure. Because of this irritability it was found essential to include a number of controls with plain paste in all these tests and to disregard the results observed whenever it was known that the sporangiophore had been comparatively roughly handled. While emphasizing these limitations and the fact that the positive results are not quantitatively consistent, it seems worth while briefly to discuss them, since their implications are of some importance.

ii. *Results with heteroauxin*

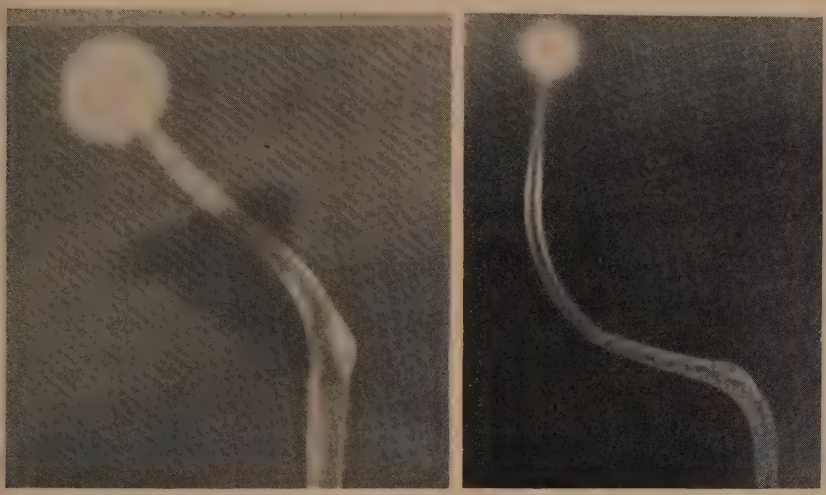
The tests with heteroauxin paste did not at any of the concentrations used provide any clear indication of a growth response; slight curvatures, variable in direction, were occasionally observed, as they also were with plain paste. In view of the range of concentration covered, 10,000–10 μg./ml., and the fact that the results were indistinguishable from those with plain paste, it is concluded that heteroauxin unilaterally applied as described to the outside of sporangiophores of *Phycomyces* has no action on their growth. This result is in harmony with the work of Brian (1949), who found that heteroauxin at low concentrations (1–10 μg./ml. in culture media) was without action on mycelial growth, while at higher concentrations it produced some stunting. It appears that despite the evidence for the occurrence of heteroauxin in fungi there is no direct reason, apart from analogy with higher plants, to suppose that it or any similar phytohormone has any growth-regulating influence on chitin-walled fungi.

Ferri's (1951) results do, however, suggest that it would be some evidence

n favour of the heteroauxin hypothesis if it could be shown that *Phycomyces* porangiophores will take up eosin and then fail to give a phototropic response.

ii. *Results with griseofulvin.*

When griseofulvin paste containing 100 μg./ml. was supplied as described, t produced a sharp curvature in the sporangiophores (Fig. 2, *a* and *b*). This end was the result of a change in direction of subsequent growth, not a local stretching of an already matured hyphal wall. Griseofulvin paste applied at



(*a*) (*b*)

FIG. 2.

points several millimetres below the growing zone produced no apparent eaction: whether this was due to the inextensibility of the older wall, its mpermeability, or the insensitivity of the cytoplasm is not known, though the irst explanation seems probable.

When the paste was applied to the growing zone the direction of the curvature with reference to the point of application varied. It may well have been nfluenced by the relative rates of elongation and of spiral axial rotation of the porangiophores, and by the rate of penetration of griseofulvin through the yphal wall. With the *Phycomyces* strains used, the bend was always away rom the point of application, though it sometimes veered to the right and losely approached a transverse direction. Beyond the griseofulvin-induced urvature the sporangiophore did not grow in a straight line; instead it continued in a very steep left-hand spiral and then curved back into a vertical irection, so manifesting the normal negative geotropic response (Fig. 2*b*).

The majority of the tests were performed on sporangiophores with terminal porangia, but sporangiophores which had not reached the stage of sporangium ormation behaved in a similar manner when unilaterally treated. When the riseofulvin paste was applied uniformly to the tips of the apical cones of such porangiophores the formation of a terminal sporangial swelling was not

induced: instead, the sporangiophores grew on in a steep morphological left hand spiral around a vertical axis (i.e. clockwise growth viewed from above). (The interpretation of spiral rotation and of the growth stages of the sporangiophore developed by Preston (1948) appears to be consistent with this observation.) Similar but less pronounced spirals were twice observed with plain paste applied to the apex, though usually this treatment was without any obvious effect.

Griseofulvin paste applied to the growing zone always modified the subsequent growth to some extent: there can be no question of its powerful physiological activity under the conditions discussed. A quantitative statement of this activity analogous to the '*Avena* Einheit' will not be readily achieved.

There is always some, often considerable, variation in diameter and growth rate between the individual sporangiophores arising from a particular slide trough, even after the more delicate ones have been plucked out. The size and growth rate of a particular sporangiophore must be greatly influenced by the extent and activity of the local area of substrate mycelium forming its absorbing system. This variability hampers a quantitative treatment of the curvatures, and a further difficulty arises in assessing the dose applied. Using micrometer eyepiece measurements followed by comparison with plasticine models, it was estimated that the volume of paste applied lay between 10^4 and 10^5 μ^3, and was usually in the lower part of that range. The limits given correspond with applied griseofulvin doses of 10^{-6} to 10^{-5} μg. per sporangiophore. It is not possible to calculate the fraction, probably small, of this dose that entered the sporangiophore, and indeed the partition coefficient for griseofulvin between water or cytoplasm and the lanolin-paraffin solution is not known. It is, however, hoped to make the results of current experiments at greater dilutions using an aqueous medium the subject of a further communication.

For the reasons outlined, observations so far obtained cannot be placed on a satisfactory quantitative basis. It can only be stated that most of the curvatures fell into the range of 30° to 80° from the vertical, but occasionally a sporangiophore was observed to loop completely round through 360°. Loops such as this were never observed with plain paste.

iv. *Phototropism*

While the quantitative aspects of the phototropism of sporangiophores have been studied in great detail, no satisfactory explanation in terms of a myco-hormone or auxin has yet been developed. If the view taken in Part I is correct, phototropism is the result of a positive light-growth reaction. Growth is here envisaged primarily as a result of increased ductility of the hyphal wall or an increased rate of intussusceptive deposition of glucosamine micelles in the wall which is under turgor tension. The action of light must then be to accelerate one or both of these processes. This might result from production, activation, or redistribution of a growth-promoting substance, destruction or inactivation of a growth inhibitor, or sensitization of the responding zone. An

xplanation based on a check to growth on the side facing the light is not dmissible on the available evidence, and for this reason it seems that Wassink nd Bouman (1947) are mistaken in speaking of the inactivation of a growth romoter. It also seems that even if Kögl and Verkaaik are quite correct in naintaining the occurrence in *Phycomyces* of auxin-*a* or whatever substance t was that they isolated with the properties they outline, then it is still not ossible to explain the phototropic response in terms of its photo-inactivation. f an analogy with the behaviour of heteroauxin (and other synthetic sub-tances having similar physiological action) described by Brian (1949) has any elevance here, it inclines one to doubt whether a substance having a growth-romoting action on the *Avena* coleoptile would be likely to act similarly n *Phycomyces*.

Griseofulvin fulfils one criterion for a myco-auxin: it influences the exten-ion of chitin-walled hyphae. It is produced by several *Penicillium* species ut has not been demonstrated in *Phycomyces* mycelium or metabolite fluid. t is not photo-inactivated *in vitro* and is indeed a very stable substance, and here is no reason at present to suppose that it is in fact concerned in the hoto-responses of *Phycomyces*. It is, however, interesting as the first instance f a chemically defined substance produced by a fungus and acting on fungal yphae in a manner analogous to the action of heteroauxin on cellulose-valled cells.

It is clearly desirable that attempts should be made to extract a myco-uxin from sporangiophores grown in the dark and then illuminated for a hort period. If a substance, perhaps obtained in this way, can be isolated nd shown to be effective in influencing the growth of sporangiophores, that vill respresent a valuable contribution to the study of growth regulation in ungi. In the meantime it would now seem rather unprofitable to erect ypotheses based on substances showing no evidence of possessing the equisite properties.

ACKNOWLEDGEMENTS

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The Absorption of Water by Leaves in an Atmosphere of High Humidity

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SUMMARY

No evidence could be obtained for any active exudation of water by the roots of tomato plants with the foliage in an atmosphere of high humidity. In as far as the plants remained healthy and grew, the absorption of water by the leaves as reported by Breazeale is confirmed.

THE experiments of Breazeale *et al.* (1950) upon tomato plants grown with the foliage in saturated air or fog and the roots in initially dry soil or initially dry flasks may have occasioned some disquiet since continued loss of water by the roots after the soil has become wet or the atmosphere in the flasks nearly saturated might at first seem to suggest an active secretion by the roots in a direction the reverse of that of the normal water current. As will be mentioned again later, however, if any such capacity actually exists for actively forcing water through the plant in the reverse direction, it is more likely to reside in the leaves than in the roots. But, to be sure of the nature of the facts first, it occurred to the writer that, inasmuch as no temperature control was mentioned and the possibilities of condensation of water merely evaporated from the root surfaces were not therefore ruled out, it might be desirable to repeat the experiments (*a*) with some precautions against temperature fluctuations that might lead to evaporation and condensation, (*b*) with special precautions against leakage of the seals or movements of water into the flasks through the intercellular space systems in response to pressure differences between the flasks and the atmosphere, and (*c*) with inanimate materials, with which there could be no question of active secretion, used instead of the plants.

As a preliminary some experiments were performed exactly after the manner of Breazeale's fourth series, the roots of tomato plants being sealed into initially dry Ehrlenmeyer flasks and the whole set-up being placed in a fogging-chamber, the tops being freely exposed to fog. Fog was obtained by means of a vaporizer worked by a compressor and $\frac{1}{4}$-h.p. electric motor. There was no temperature control. The results were very similar to those of Breazeale, so need not be given in detail. In the course of fifteen experiments lasting from 10 to 30 days, amounts of water collected in the flasks varying from 17 to 150 ml., the amounts appeared superficially, by eye estimates only, to be related to the bulk or surface of the roots systems, and especially to the quantity of new and younger roots present. Younger roots also remained moist while older roots became dry.

It seemed that the results might easily be accounted for by changes in temperature of the flasks. Normal evaporation from the moist root surfaces

would be expected to occur as long as the air in the flasks was not quite saturated. (Even if the air were saturated the process might continue through the root temperature being slightly above that of the air as a result of respiration.) On cooling at night some condensation would take place. On warming the next day the saturation deficit would increase, but the higher heat capacity of the water already condensed would lead to a lag in its heating up and its re-evaporation would be delayed, although, the younger roots having smaller heat capacities and therefore warming up more rapidly, renewed evaporation would take place from the root surfaces. Repetition of the process would lead to further collection of water in the flasks at rates depending on the heat capacity of that already collected and therefore increasing with time. Water might also be forced into the flasks through flaws in the seal or through the intercellular space system when the internal pressure fell with a fall in temperature. That most of the water appeared to collect at night would support either of these suggestions.

To determine whether the water collected was in fact exuded (or whether it entered) as liquid, as opposed to being evaporated and recondensed, experiments were next carried out with the roots in separate small specimen tubes suspended inside the flasks. In these experiments in most cases large quantities of water collected in the flasks as before, but usually none or only traces in the specimen tubes. This meant that the water in the flasks could not have come as liquid water from the roots but could only have reached the flasks by condensation of vapour. In the few cases where 10–15 ml. of water collected in the tubes it appeared to have been forced in via the intercellular space system by changes in pressure.

In a third series of experiments, to eliminate this last-mentioned possibility also, a special fog chamber was constructed having a removable glass top and sides and a sheet metal base, 3 ft. by 15 in., perforated by 25 evenly spaced 1-in. holes. The base of the chamber was supported about 8 in. above the greenhouse bench and twenty-five Ehrlenmeyer flasks were placed beneath it with their bases partially embedded in damp shingle to avoid any rapid fluctuations in temperature. Tomato plants were sealed by split cork and wax seals through the holes in the floor of the chamber with their tops in the chamber which was filled with fog by the vaporizer. Their roots were placed in the separate dry Ehrlenmeyer flasks, the necks of which were well plugged with cotton-wool. The replacement of wax seals to the flasks by cotton-wool plugs eliminated the tendency of changes in pressure to force water of surface films through the intercellular space systems or elsewhere into the flasks.

An experiment of this type was maintained for 3 weeks with twenty-five tomato plants. Except in three cases, in which some water collected as a result of defective seals and by running through the cotton-wool plug, no appreciable quantity of water collected in any of the flasks. Only some mist collected on the sides of the flasks, obviously as a result of condensation, and never enough to run down or collect in the bottoms of the flasks. There was thus never enough to be regarded as more than a trace due to condensation of

water evaporated in the normal way from the moist root surfaces. No evidence for any active secretion could be obtained. In these circumstances there was no continued transfer of water from the foliage to the flasks.

In case these results might be due to low metabolic state through lack of nutrients, a further twelve experiments were carried out with a layer of soil 2 in. deep surrounding the lower parts of the stems in the fogging-chamber. New roots were formed and grew out into this soil, but after 15 days there was still no transfer of water to the flasks.

In a number of experiments in which a piece of string or wick was set up in exactly the same way as the tomato plants in Breazeale's fourth series and in the writer's preliminary experiments, with one end in an initially dry flask and the other in water or in saturated air or fog, results were obtained which were precisely similar to those of Breazeale with plants. The only difference was that with an inanimate wick rates of collection of water up to ten times the greatest recorded with tomato plants were obtained. This appeared finally to dispense with the necessity of invoking active secretion by roots into saturated or nearly saturated air to explain the results obtained by Breazeale and by the writer in the experiments under totally uncontrolled conditions of temperature and with sealed flasks allowing considerable fluctuations of pressure relative to that of the surrounding atmosphere.

It would appear (with one possible exception to which return will be made below) that there is nothing unexpected about any of the results. The plant by reason of its capacity for automatically setting up a gradient in suction pressures between the source of water-supply and the sink would be expected to convey water from any point of higher diffusion potential to lower. The experiments in which water is conveyed to dry soil are all explicable on this principle. The explanation of those in which water is transferred to a closed flask (in so far as this occurs) may be less obvious, but, inasmuch as the atmosphere round the roots has not in any experiment been kept continuously as nearly saturated as that round the leaves, there would naturally be more normal (net) evaporation into the atmosphere round the roots than into that round the leaves, and by purely physical conditions a reversed conduction current would be automatically set up.

The possible exception mentioned above is the experiment of Breazeale in which adventitious roots raised the water-content of a column of soil in a tube round the base of the stem to a higher value when the plant was supplied with water as fog via the leaves than when supplied via the roots from a well-watered soil. This experiment is of great interest but does not necessarily provide any evidence for secretion of water by the roots. Since both the fogged atmosphere and the well-watered soil virtually represent saturation, water is only passing down its own free energy gradient in each case. The difference might well be due to differences in the resistances encountered on the two paths. If, however, any secretion or active transfer of water should be responsible for the greater supply from the leaves than from the roots, it would appear on the face of it much more likely that the leaves in fog actively

absorb than that the roots in soil actively secrete. Such a hypothetical secre tion by the roots would only go to aggravate the plant's problem of maintain ing a workable water-content against the tendency to evaporation, but tendency of the leaves to bring about a secretory or active inward or down ward movement of water would help to lessen the general problem and assis in acting as a check to water loss. It appears then that Breazeale's results pro vide little ground for suspecting any active secretory capacity on the part o the roots, but that they may have a special significance in connexion with th possibility of active absorption by the leaves. This aspect of the problem woul probably repay further investigation.

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Studies in the Principles of Phytotoxicity

III. THE pH FACTOR AND THE TOXICITY OF 3:5-DINITRO-*o*-CRESOL, A WEAK ACID

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SUMMARY

It has previously been shown that the concentration of 3:5-dinitro-*o*-cresol required in the external medium to halve (*a*) the growth rate of the mould *Trichoderma viride* or (*b*) the respiration rate of yeast increased some 75–100 times as the pH changed from 4 to 7. In the present investigation it has been found that when solutions or suspensions of 3:5-dinitro-*o*-cresol are applied as sprays to seedlings of *Brassica alba* the concentration needed to kill half the plants changes by a factor of less than 2 over the same pH range.

On the other hand, when *Lemna minor* is grown in nutrient solution containing the toxicant, the concentration required to halve the rate of frond multiplication increases sharply as the pH is raised from 5·4 to 6·8. Moreover, when leaf disks of *B. alba* are vacuum infiltrated with buffered solutions of 3:5-dinitro-*o*-cresol the concentration needed to halve the respiration rate rises 50-fold between pH 4 and 8.

It is considered that the essential condition which determines the similarity of the results for *L. minor*, the leaf disks, and micro-organisms is that a relatively large volume of solution containing the toxicant is in direct contact with the tissues. In contrast, under the conditions of spraying the volume of spray droplets relative to that of plant tissue is small. Thus the pH effect is masked since the buffering capacity of the cells exceeds that of the droplets, with the result that 3:5-dinitro-*o*-cresol largely acts upon the cells at a pH determined by the tissues, a pH which may bear little relation to that of the original solution.

Supporting evidence is produced that the pH factor operates in the same way for other weak acids, such as the chlorophenoxyacetic acids.

INTRODUCTION

IT has now been established that the toxic or other biological effects of weak acids on bacteria and fungi are greater in acid than in alkaline solutions (Simon and Blackman, 1949; Simon and Beevers, 1952*a*). For example, in the investigation concerned with the relationship between pH and the toxicity of nitro-phenols to fungi it has been shown that at pH levels where there was much dissociation the toxicity fell sharply. Thus when the pH of a solution of 3:5-dinitro-*o*-cresol—which has a pK of 4·4—was changed from 4 to 7 the concentration required in the agar medium to halve the growth rate of the mould *Trichoderma viride* was increased by 75 times. Similarly, for a corresponding change in pH level the concentration needed to halve the respiration rate of yeast was increased 100 times.

3 : 5-dinitro-*o*-cresol and a number of other weak acids, such as the chloro phenoxyacetic acids, are widely used in the field as selective herbicides and the question therefore arises whether their toxicity is influenced by the pH of the spray solution. In the case of 3 : 5-dinitro-*o*-cresol some indication of the effect of pH can be obtained from field experiments in which comparisons are made between spray suspensions prepared from the acid and solutions made up from the sodium salt, since a suspension of the acid at 0·6 g./100 ml. —which would give a spray of normal field strength—has a pH of 4·2, whilst a solution of the same strength prepared from the sodium salt has a pH of 7·3. The results of field experiments (Blackman, 1945 ; Blackman *et al.*, 1949) show that the acid is usually more toxic than the sodium salt, but that the difference seldom exceeds two or three times. In addition, it may be noted that the increase in toxicity which follows the addition of ammonium sulphate to sodium dinitro-*o*-cresylate is sometimes greater than could be ascribed solely to the shift in pH (Blackman *et al.*, loc. cit.).

From this and other evidence of field trials it seemed that the pH factor was of far less importance than in the case of fungi and bacteria. It must, however, be borne in mind that the conditions were widely different. In the experiments with *T. viride* and yeast there was a large volume of external medium containing 3 : 5-dinitro-*o*-cresol, while in the field the material was applied as a fine spray on to the shoots. Again the seedlings investigated differed from the lower organisms in their far more complex structure and in their metabolic and photosynthetic processes. The present investigation has been planned against a background of these considerations.

In the first paper of this series (Blackman, 1952, p. 1) it has been pointed out that in studies of toxicity *Lemna minor*, a higher plant with a relatively simple structure, is a valuable link between unicellular organisms and other higher plants. It has also been emphasized that experiments with isolated pieces of tissue, such as leaf disks, should be complementary to greenhouse and field experiments. Lastly, because the relationship between the dose of 3 : 5-dinitro-*o*-cresol and the response is S-shaped the greatest precision is achieved by assessing toxicity in terms of the amount which halves the effect under investigation (Sampford, 1952).

EXPERIMENTAL METHODS

Spraying experiments with seedlings of Brassica alba

Pots of seedlings of *Brassica alba* L. were sprayed in a greenhouse under conditions resembling those of field practice. The spray treatments were applied when the plants were about 5 cm. high and had two true leaves larger than the cotyledons. The spraying apparatus consisted of a moving trolley carrying a glass spray reservoir, spray arm, and a nozzle delivering a flat fan spray. The reservoir was connected to an air compressor, adjusted so that a constant pressure of 50 lb./sq. in. was maintained. The speed of the electric motor driving the trolley was regulated by a rheostat so that as the nozzle

moved over the pots the rate of spray delivery was equivalent to 100 gallons per acre.

Experiments were carried out with this apparatus to determine the effect of varying the pH of the spray. In each experiment a range of concentrations buffered with M/15 phosphate was employed and each concentration was applied to 3 pots containing in all about 50 plants. Some 10 days later the number of seedlings surviving in each pot was counted. For each pH level the percentage mortality was plotted against the logarithm of concentration and a sigmoid curve fitted to the points by eye. From the curves the concentrations required to bring about a 50 per cent. mortality were determined.

Reduction in the growth rate of Lemna minor

A clonal strain of *Lemna minor* L. was grown in the simple inorganic medium described by Clark (1926). Glass-distilled water was used throughout and M/150 phosphate added to act as a buffer. The culture vessels employed were 250-ml. squat Pyrex beakers immersed in a rectangular water-bath at a controlled temperature of 25° C. Continuous illumination of approximately 200 foot-candles was supplied by a fluorescent 'daylight' tube and under these conditions a healthy and consistent growth was obtained in the controls.

At the start of each experiment a range of concentrations of 3 : 5-dinitro-*o*-cresol was made up in the culture medium and three replicate beakers prepared for each concentration. Plants of *L. minor*, randomly selected from the stock culture, were put into the beakers so that each contained 20 fronds. These beakers, together with the controls, were placed in the water-bath and randomly arranged in three longitudinal blocks. In order to minimize changes in pH, solutions were renewed and the positions of the beakers re-randomized within blocks every 48 hours.

It has been pointed out (Blackman, 1952) that with non-lethal concentrations of dinitro-alkyl phenols changes in the dry weight of *L. minor* are closely correlated with change in frond number. Increase in frond number was therefore taken as the measure of growth, daughter fronds being excluded from the counts until they attained a diameter of 1 mm. The duration of each experiment was 7 days, daily counts being made from the 3rd to the 7th day inclusive. The growth of a normal colony of *L. minor* as measured by increase in frond number is exponential, so that when the logarithm of frond number is plotted against time, there is a linear relationship. If a linear regression be calculated for such data, the regression coefficient will be a measure of the growth rate of the colony.

It was found that the growth of colonies of *L. minor* in solutions containing sub-lethal concentrations of 3 : 5-dinitro-*o*-cresol is also logarithmic, but that the growth rate is reduced (Fig. 1A). Regression coefficients were therefore calculated for each treatment and expressed as percentages of the regression coefficient for the controls. These percentage values were plotted against the logarithms of the concentrations and from the resulting S-shaped curves (Fig. 1B) the concentrations required to halve the growth rate were determined

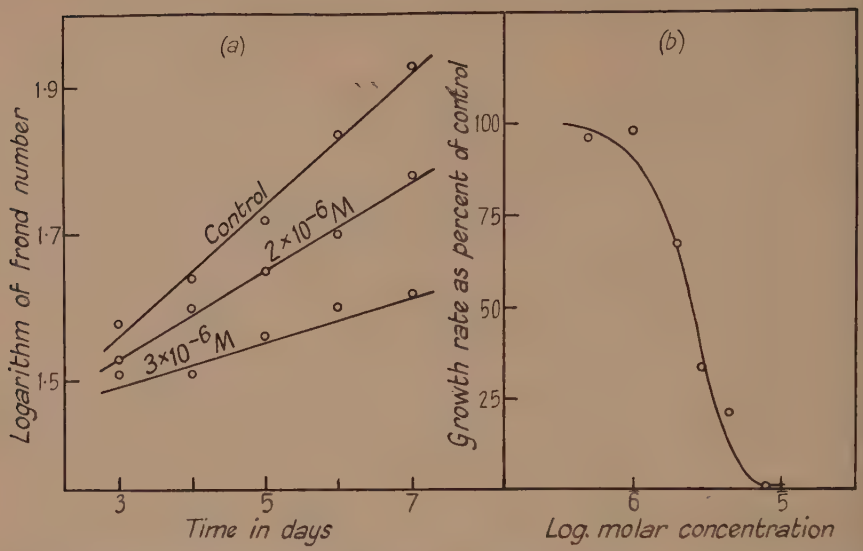

FIG. 1. A. The effect of various concentrations of 3:5-dinitro-*o*-cresol on the frond number of *Lemna minor* at pH 5·4. B. The effect of increasing concentrations of 3:5-dinitro-*o*-cresol on the rate of increase of frond number of *L. minor* at pH 5·4.

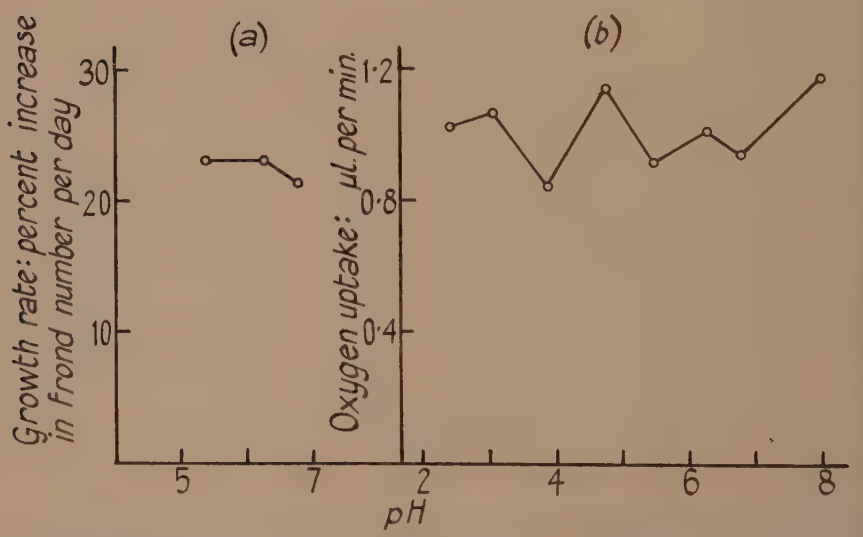

FIG. 2. A. The effect of pH on the growth rate of *L. minor*. B. The effect of pH on the respiration rate of infiltrated leaf disks of *Brassica alba*.

by interpolation (cf. Sampford, 1952). Experiments were carried out at three pH levels between 5 and 7, and within this range there was little variation in the growth rate of the controls; see Fig. 2A.

Respiration rate of infiltrated leaf disks of Brassica alba.

The seedlings of *B. alba* chosen for these experiments were at the same stage of growth as those used in the spraying experiments. Disks of 8·5 mm. diameter were cut out of the true leaves with a cork borer and placed in Warburg manometric vessels. Care was taken to avoid an accumulation of disks from the leaves of one plant in one vessel by placing consecutive disks in the different vessels in rotation until each had 15 disks. This procedure ensured that each vessel had as nearly as possible the same amount of respiring

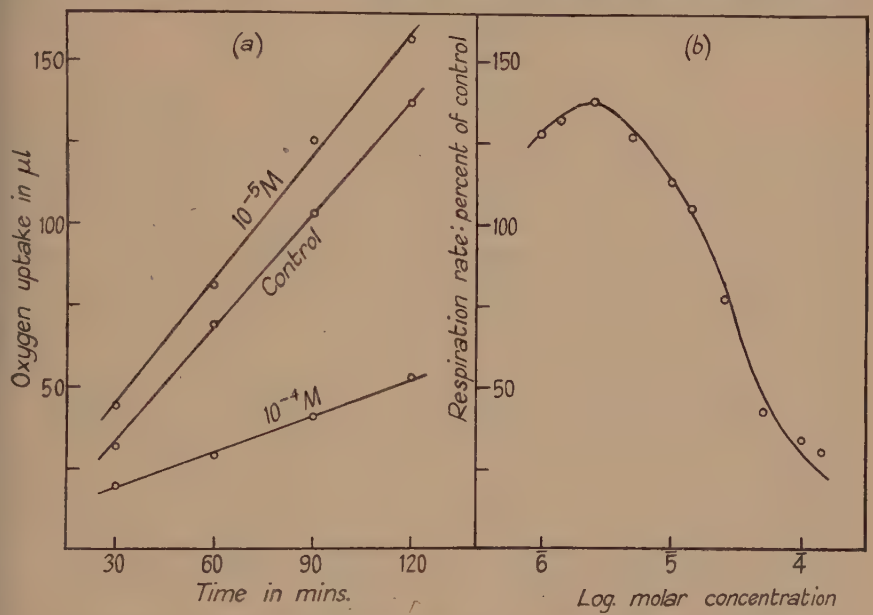

FIG. 3. A. The effect of various concentrations of 3:5-dinitro-*o*-cresol on the oxygen uptake of leaf disks of *Brassica alba* at pH 4·8. B. The effect of increasing concentrations of 3:5-dinitro-*o*-cresol on the rate of respiration of leaf disks of *B. alba* at pH 4·8.

material. Thus in a control experiment with 13 replicates, the coefficient of variation of the rate of oxygen uptake was found to be 7·5 per cent., a value which was considered satisfactory. Experiments on the effect of pH were carried out over the range of pH from 2·5 to 8, and as can be seen from Fig. 2B the respiration rate of the controls showed no systematic trend.

The vessels contained 2 ml. of M/15 phosphate buffer, adjusted to the desired pH, and 0·5 ml. of the appropriate solution of 3:5-dinitro-*o*-cresol or of distilled water. The solution in each vessel was vacuum infiltrated into the disks, usually by means of five or six short evacuations, although subsequent experiments have shown that two evacuations of longer duration are equally satisfactory. The rate of oxygen uptake was measured by the conventional manometric method at 25° C. The rate was constant over the period of the experiments (Fig. 3A) and was computed from measurements taken at four intervals of half an hour.

From Fig. 3B it is evident that oxygen uptake is stimulated by low concentrations of 3 : 5-dinitro-*o*-cresol although higher concentrations cause an inhibition. A similar graph showing both stimulation and inhibition of the oxygen uptake of yeast was published by Simon and Beevers (1952*a*, Fig. 5A), and several authors have reported that appropriate concentrations of nitrophenols stimulate the respiration of other plants and animal tissues (cf. review by Blackman *et al.*, 1951). For the purpose of this investigation the criterion of toxicity adopted was the concentration required to halve the respiration rate at each pH and these values were obtained by interpolation from the corresponding dosage-response curves.

EXPERIMENTAL RESULTS

Using the spraying technique already described, experiments were carried out in which 3 : 5-dinitro-*o*-cresol was applied to seedlings at a range of concentrations and at four levels of pH. It is evident from the results (Fig. 4) that a change of pH has only a small effect on toxicity. The concentration required to bring about a 50 per cent. mortality of *B. alba* seedlings at pH 4 was $1{\cdot}2 \times 10^{-3}$ M., while the corresponding concentration at pH 8 was $1{\cdot}7 \times 10^{-3}$ M. Another experiment in which unbuffered sprays were used gave very similar results.

In the *L. minor* experiments, by contrast, change of pH had a considerable influence on the concentration required to halve the rate of frond multiplication. From Fig. 4 it is apparent that with a change of pH from 5·4 to 6·8 there has been an 8-fold increase in the equi-effective concentration.

It has already been emphasized that the conditions of spraying are different from those of experiments in which the organism is in permanent contact with a large volume of solution containing the toxicant. In the case of *L. minor* the plants float on the surface of the medium and the lower surfaces of the fronds and the buds are less heavily cuticularized than the aerial parts of mustard seedlings. Hence the toxic solutions will probably penetrate more readily to the individual cells of the fronds than into the leaves and stems of *B. alba*.

Under the experimental conditions with leaf disks, as a result of vacuum infiltration, the toxic solution is in even more intimate contact with the individual cells than with *L. minor*, and it will be seen (Fig. 4) that the change of pH has a large influence on toxicity in this case also. The concentration needed to reduce the respiration rate of the leaf disks by 50 per cent. increases by about 50 times as the pH is changed from 4 to 8.

DISCUSSION

The present experiments have demonstrated that change of pH has a marked effect on the concentrations of 3 : 5-dinitro-*o*-cresol required to halve the growth rate of *L. minor* or the respiration rate of infiltrated leaf disks. It can be seen from Fig. 4 that, over the pH range from 4 to 8, toxicity decreases by about 3 times for each unit increase of pH, a figure which is typical of the effect of pH on the toxicity of a weak acid over the pH range in which it is

much dissociated (Simon and Beevers, 1952*a*). An example from the investigations of Simon and Beevers is included in Fig. 4 by way of comparison; the curve for the effect of pH on the concentrations of 3:5-dinitro-*o*-cresol required to halve the respiration rate of yeast is very similar to that for the leaf disk experiments.

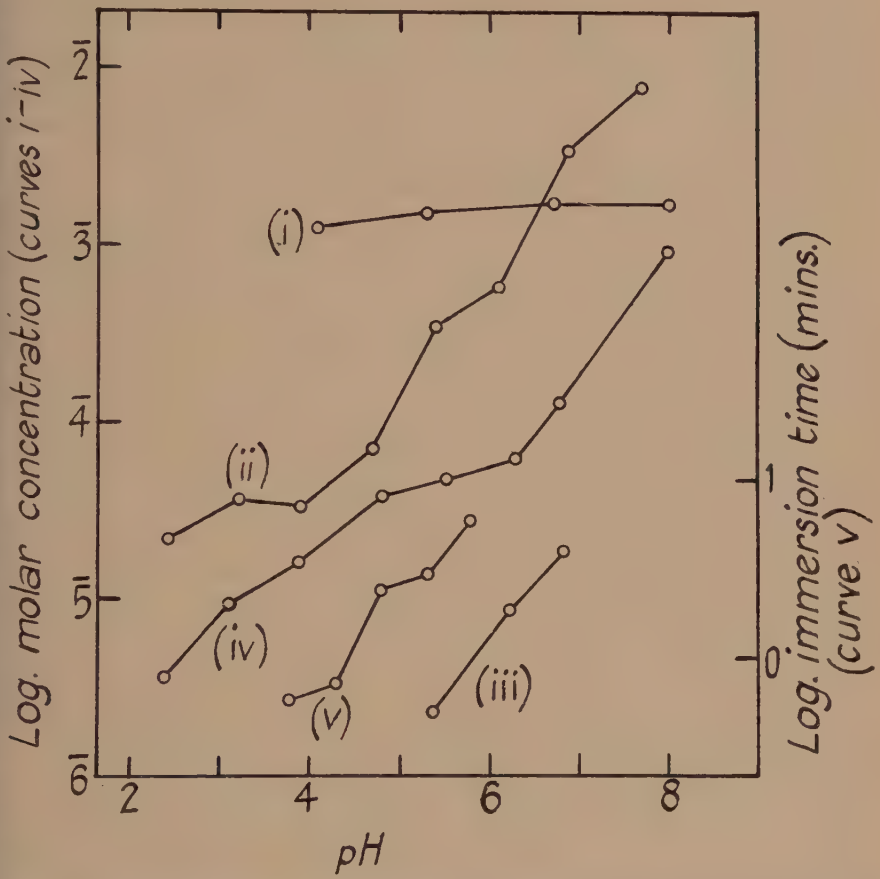

FIG. 4. The effect of pH on the concentrations of 3:5-dinitro-*o*-cresol required for (i) 50% mortality of seedlings of *B. alba*, (ii) 50% reduction in the rate of respiration of yeast (data of Simon and Beevers, 1952*a*), (iii) 50% reduction in the growth rate of *L. minor*, and (iv) 50% reduction in the respiration rate of infiltrated leaf disks of *B. alba*. (v) The effect of pH on the time of immersion in 0·01% 3:5-dinitro-*o*-cresol required for 50% kill of leaves of *Sinapis arvensis* (data of Fogg, 1948).

A further example can be taken from the work of Fogg (1948) on the penetration of 3:5-dinitro-*o*-cresol into leaves of *Sinapis arvensis*. Leaves were dipped for various lengths of time into buffered 0·01 per cent. solutions, subsequently removed, washed, and allowed to stand in a cool greenhouse for 2 days, when the proportion of tissue killed was determined. Fogg showed that the quantity of 3:5-dinitro-*o*-cresol taken up by leaves was proportional

to the time of immersion, so that the time required for 50 per cent. kill could be used as a measure of toxicity at the pH of the solution. The results are shown in Fig. 4 as a graph of the logarithm of the immersion time for 50 per cent. kill against pH, and it will be seen that in this instance, again, toxicity falls by about 3 times per unit increase of pH.

The pH experiments with *L. minor* and with leaf disks reported in this paper, and the leaf-dipping experiments of Fogg, therefore form a consistent body of data, comparable in every way to the ninety pH experiments with various weak acids and test organisms that were discussed by Simon and Beevers (1952*a*). The essential condition common to all these experiments is that a relatively large volume of solution containing the weak acid is in intimate contact with the cells of the tissues.

On the other hand, it will be recalled that change of pH had very little effect on the toxicity of 3 : 5-dinitro-*o*-cresol sprays to seedlings of *B. alba*. The reason for this becomes clear when the results are considered in conjunction with those for leaf disks, *L. minor*, &c. When solutions or suspensions of 3 : 5-dinitro-*o*-cresol at a particular pH are sprayed on to the shoots of seedlings the toxic material may penetrate to the epidermal cells at the pH in question, but the more distant cells in the bulk of the tissue are not in contact with the original solution. Thus the pH of the toxic material which passes from cell to cell within the plant is likely to be determined by the pH of the cell contents and may bear no relation to the pH of the original spray solution. Therefore changes in the pH of the spray have little or no effect on toxicity.

The fact that 3 : 5-dinitro-*o*-cresol is slightly less toxic to seedlings under alkaline than acid conditions (*vide* Fig. 4) suggests that the pH factor is not entirely inoperative. However, there are other factors which must be taken into account in discussing the relative toxicity of the acid and its sodium salt.

It has been pointed out (Blackman, 1950, 1952) that under the conditions of spraying the quantity of toxicant entering the tissues will depend on retention by the shoot and penetration into the tissues and that both these factors will be influenced by the physico-chemical properties of the spray droplets. At the concentrations used in the field—0·2–0·8 g. per 100 ml.—the sodium salt of 3 : 5-dinitro-*o*-cresol is wholly soluble but the acid is in the form of a suspension and the presence of solid particles in the spray may increase the area of leaf surface covered by the individual spray droplets (Fogg, 1948). On the other hand, because of the greater solubility of the sodium salt the spray droplets will take longer to dry out on the shoot surfaces. In addition, Fogg (1948) has demonstrated experimentally that 3 : 5-dinitro-*o*-cresol can enter the leaf tissues of *Sinapis arvensis* by diffusion through the stomata, and as the vapour pressure of the acid is very much greater than that of the sodium salt, the acid is more likely to penetrate in this way. Moreover, the acid will be more lipoid soluble and may be more freely taken up by the cuticle. It follows that the differences in toxicity between the sodium salt and the acid may depend on differences in spray retention and penetration as well as pH.

From the foregoing discussion it is not to be expected that pH changes will

have much influence on the toxic effects of other weak acids used as herbicides. Spraying experiments, similar to those already described, have been carried out with 2:4-dichlorophenoxyacetic and 2-methyl-4-chlorophenoxyacetic acids. Sprays buffered with phosphate to pH 4, 6, and 8 were applied to seedlings of *B. alba* and the concentrations required for 50 per cent. mortality showed a negligible variation with pH. On the other hand, in experiments on *Linum usitatissimum*, to be reported in full elsewhere, some indication has been obtained that 2:4-dichlorophenoxyacetic acid is slightly more toxic than its sodium salt, but here—as in the case of 3:5-dinitro-*o*-cresol—difficulties of interpretation arise since the acid is relatively insoluble in water and was applied as a suspension.

Under conditions in which growth substances are applied in a large volume of solution to a relatively small amount of tissue, e.g. in the split-pea test and the *Avena* cylinder growth test, change of pH has a considerable influence on activity. The effect of pH is, however, masked in tests where a small amount of material is applied to a relatively large test object, as in the *Avena* curvature test and the leaf epinasty test (Simon and Beevers, 1952*b*).

With the chlor-substituted phenoxyacetic acids there is some evidence that under the appropriate conditions activity is influenced by pH. Stenlid (1949) found that a 0·001 M. solution of 2:4-dichlorophenoxyacetic acid at pH 4·5 gave 80 per cent. inhibition of the respiration rate of wheat roots while at pH 7 there was no inhibition at all with a concentration twice as great. Audus (1949) records that the percentage inhibition of the root growth of *Lepidium sativum* by 0·2 p.p.m. of the same acid dropped from 95 per cent. at pH 4·5 to 40 per cent. at pH 8. In addition, preliminary experiments by R. C. R. Cuninghame in this laboratory have indicated that the toxic action of 2:4-dichlorophenoxyacetic acid to *L. minor* is influenced by pH in the same way as that of 3:5-dinitro-*o*-cresol.

The data for the growth regulators so far presented are in close accordance with the findings for 3:5-dinitro-*o*-cresol, but it must be noted that workers from Michigan (Lucas and Hamner, 1947; Hamner *et al.*, 1947; Lucas *et al.*, 1948) report experiments from which it was inferred that changes in the pH affected the activity of 2:4-dichlorophenoxyacetic acid to seedlings. Single drops of a solution of the acid at 1,000 p.p.m. were applied to the primary leaves of *Phaseolus vulgaris* and it was claimed that the amount of subsequent growth depended on the pH of the drop and also on the nature of the buffer used. It is difficult to interpret the results of experiments in which a single concentration alone is used (Blackman, 1952; Simon and Beevers, 1952*a*), and it is not clear why the acid used to acidify the solutions and the presence or absence of buffer should have had so large an influence on the results obtained. Possibly other factors than pH were involved, perhaps variations in the physico-chemical properties of the spray droplets affecting the area of contact, the rate of drying out, and hence the amount entering the tissues.

On the basis of the present results it can be concluded that where weak acids are applied as herbicidal sprays the effect of pH will be small. But the pH factor

may be of importance for the control of aquatic vegetation, especially for species which are wholly submerged or have floating leaves, since the tissues will then be in contact with a large volume of water containing the toxicant. In fact, for species with a relatively simple structure the variation in the toxic effect with pH should equal that found for *L. minor*. From the point of view of practice it is unfortunate that the waters most likely to support a dense vegetation have a high base content and a neutral to slightly alkaline reaction since weak acids such as 3:5-dinitro-*o*-cresol or the chlor-substituted phenoxyacetic acids, will be operating relatively ineffectively under such conditions. It follows that if a choice exists between phytotoxic weak acids, a compound which has a relatively high pK should be selected so that it will be little dissociated under the conditions of use. On the other hand, a basic compound would be most effective in neutral or alkaline waters, while the toxicity of a non-electrolyte should be independent of the pH of the water.

Lastly, there remains for discussion whether the great variation in the resistance of species to weak acids such as 3:5-dinitro-*o*-cresol or 2:4-dichlorophenoxyacetic acid is related to differences in the internal pH of the tissues. Clearly there is no simple relationship, since some species with acid saps, such as *Rumex acetosella*, are relatively resistant to the chlorophenoxy-acetic acids, while others like *Vitis vinifera* are extremely susceptible. It must be pointed out, however, that the toxic action which results in death almost certainly takes place in the cytoplasm and that in the last analysis it is the appropriate cytoplasmic pH with which resistance should be correlated. Further consideration along these lines will only be possible when more is known of the nature of the cytoplasm and the mode of action of the individual compounds.

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Studies in Stomatal Behaviour

II. THE ROLE OF STARCH IN THE LIGHT RESPONSE OF STOMATA

Part 3. Quantitative Relationships in Pelargonium

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(*Received 29 May 1951*)

SUMMARY

The relationship between starch-content and aperture in the stomata of *Pelargonium* has been investigated by a quantitative technique. Heath's suggestion of an inherent diurnal rhythm in starch-content is confirmed, and the light effect which has been the subject of previous contradictory reports is found to be dependent on external humidity. When humidity is high, light (which in these experiments is confounded with reduced CO_2-content) causes a striking reduction in stomatal starch; when humidity is low, light has no effect on starch, but its effect on aperture is unchanged. No evidence for any dependence of aperture on carbohydrate status was obtained, and it is suggested that the function of carbohydrate changes in stomata is, as earlier suggested by Kisselew, the amplifying and stabilizing of changes primarily controlled by other factors.

INTRODUCTION

The comprehensive review and discussion of the literature with which Heath (1949) opened this series makes any historical introduction unnecessary. The situation after that paper had been published can be briefly summarized as follows:

(i) Although the literature abounds in statements to the effect that stomatal opening is accompanied by a reduction in stomatal starch, the agreement is seldom as exact as simple theory would demand.

(ii) Insufficient consideration has been given to the possibility of an inherent diurnal rhythm in starch-content. In *Pelargonium* Heath found no convincing evidence of a light effect when diurnal effects were eliminated.

(iii) In some species of *Allium* the stomata apparently respond to light although starch is never formed; Heath's investigations into the stomatal behaviour of *Allium* sp. constitute Part 2 of the present series (Heath, 1951).

Small (1950) has pointed out that if there is in fact a causal relationship between the carbohydrate status of the stoma and its degree of opening, this must presumably depend on the sugar concentration, the starch representing excess carbohydrate. Such a view has two inescapable conclusions. First, under any one set of external conditions, the starch-content of the guard-cells

might be expected to be distributed in a more or less random manner over the surface of the leaf. From evidence which will be presented in a later paper, I believe this to be true. Secondly, alteration in mean stomatal aperture over a leaf, brought about by exposure to light or darkness, ought to be accompanied by a change in mean starch-content of the stomata. The difficulty in ascertaining whether this is true has always been to find any objective means of estimating this mean starch-content; to estimate the mean of a population by looking at it is seldom satisfactory. Small's own observations (Small *et al.*, 1939, 1942) refer primarily to buffer-induced movements, and there is some evidence (Williams and Shipton, 1950) for believing that such movements may be due to factors other than those operating in the normal light response of intact leaves. Alvim (1949) states in passing that *Pelargonium* shows a striking starch reduction in the light, but Heath's very extensive observations do not seem to support this.

The present investigation is a closer examination of the relation between starch and stomatal aperture in the intact leaves of *Pelargonium* under varying external conditions, made possible by the use of a quantitative technique which has already been the subject of a preliminary note (Williams and Spencer, 1950). Two types of information have emerged. First, that relating to the nature and extent of the variation between stomata of the same or different plants at any one time under any one set of conditions; this information is somewhat specialized, being primarily of interest to those actually working in the field of stomatal physiology, and for this reason it will be segregated into a separate paper (Part 4 of this series). Secondly, that relating to the changes in any one leaf when the external conditions are varied; this is the main question in dispute, and forms the subject-matter of this paper.

EXPERIMENTAL METHODS

1. *The technique of measurement*

The basis of the method is the use, as a measure of starch-content, of the *area* of the starch-grains when projected on to a plane parallel with the epidermis. The clusters of starch-grains in the guard-cells are so irregular that the area thus obtained can clearly bear no simple relation to the volume of starch present; but the method nevertheless provides a numerical 'score' for an individual guard-cell which is almost completely objective, can be associated with a measure of the aperture of the same stoma, and, in combination with other similar values, can be subjected to statistical treatment. The most likely cause of bias lies in the inadvertent selection of the stomata to be measured; for this reason the bulk of the measurements in this investigation have been carried out by an assistant who was given no information as to the treatments used, the epidermal strips being identified solely by arbitrary serial numbers. A feature of the method which adds to its trustworthiness is its tendency to minimize differences in starch-content; for the less starch there is present, the more widely separated are the individual grains, greater weight

being thereby given to small grains which would certainly be overlooked in a guard-cell rich in starch. The details of the method can conveniently be considered in three stages.

Stage (i). *Preservation and mounting*

Strips of epidermis were plunged as quickly as possible into absolute alcohol (actually 74 O.P. methylated spirit) as first suggested by Lloyd (1908), then stored in alcohol in stoppered specimen tubes. When required for measurement, the strip was transferred to a microscope slide and a drop of Heath's reagent (Heath, 1947) and a coverslip added. Excess reagent was removed by means of a filter-paper applied to the edge of the coverslip; this had the incidental advantage of flattening the epidermis if it was appreciably buckled.

Stage (ii). *Preparation of drawings*

(*a*) *Photographic method.* The method used for the early experiments has been described in outline by Williams and Spencer (1950). Individual stomata were photographed, using a $\frac{1}{7}$-in. dry objective and a Leica camera with an eyepiece viewing attachment, on to Kodak 'Microfile' film; the film was then developed in a hydroquinone-KOH mixture to give as high a contrast as possible. It was originally hoped that the resulting negative image could be projected on to a photo-electric cell, so that the area of starch—now theoretically represented by transparent patches on an opaque ground—could be rapidly measured. This was found to be quite impracticable; many trials showed that although something approaching the ideal contrast could be obtained for any one stoma, the variation in optical conditions over the strip made it impossible to select an exposure time and illumination intensity applicable to the strip as a whole. The negatives were therefore mounted in a Leica enlarger and projected on to paper to give an overall magnification of the stoma of 2,500 linear diameters. The starch-grains and aperture were then traced in outline on the paper.

(*b*) *Direct method.* There are two strong objections to the photographic method—its slowness and the difficulty of selecting an appropriate focus. Admittedly *Pelargonium* stomata are shallow, and a $\frac{1}{7}$-in. objective provides sufficient depth of focus to bring most of the starch into adequate focus simultaneously; but the aperture, conventionally measured at its narrowest point, requires a slightly different focus. It was therefore necessary either to take two photographs of each stoma at different levels (which made the method even slower) or to select a compromise focus.

Direct projection of the original mount was clearly the only satisfactory solution, since the image could then be refocused during drawing. The ordinary laboratory microprojector is not normally intended for so high a magnification, and some difficulty was experienced in obtaining an adequate light intensity; however, by using a $\frac{1}{12}$-in. oil-immersion objective and a $\times$ 15 eyepiece, a magnification of 2,000 linear diameters at an intensity adequate in a darkened room was at length achieved. As before, the starch and aperture were traced

n outline on to white paper, the drawings being suitably numbered for identification purposes. This method was used in all cases where the photographic technique is not specifically mentioned.

Stage (iii). *Measurement*

The individual areas on each diagram were joined to give a continuous figure with as few side-branches as possible; a convenient point was selected for the start, and the outline then traced with a planimeter. For the photo-

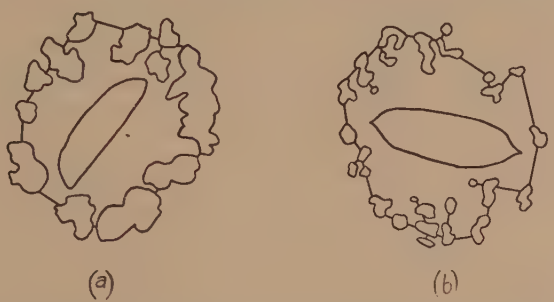

FIG. 1. Two examples of completed records. Measured areas as follows: (*a*) S = 218, A = 60; (*b*) S = 86, A = 92.

graphic records (× 2,500) the planimeter was adjusted so that it read the original area directly in square μ; for the direct records (× 2,000) the resulting planimeter setting was awkward, and it was therefore reset so that it gave this value after doubling. The planimeter readings, even for the more complex diagrams, were found to be reproducible to within a few per cent., which is certainly well within the errors inherent in the technique. Two extreme examples of the type of diagram obtained are shown in Fig. 1.

2. *General*

All experiments have been carried out on attached leaves of plants of *Pelargonium zonale* var. 'Paul Crampel' grown in a greenhouse in the Bedford College Botany Garden. In general, the leaf selected for experimental use was the first fully expanded and freely exposed leaf on the plant—usually the 3rd or 4th from the apex. Strips were taken from the lower epidermis unless otherwise stated, where possible from the outer margin of the leaf remote from the petiole. On each strip 20 stomata were selected at random and measured; the figures quoted in the tables in the body of this paper refer to means or totals, in square μ, of the areas of starch and aperture for the 20 stomata of the strip. It may be noted in passing that the statistical analyses have frequently shown a rather low variance within strips, and a rather high error variance between strips, from any one leaf; the causes of this phenomenon will be discussed in a later paper, but it may be suggested that in any similar work it would be desirable to take more strips, even at the price of counting only half

the number of stomata. References in the statistical analyses to 'significant' or 'highly significant' effects imply probability levels of 0·05 and 0·01 respectively.

All times are in G.M.T.

EXPERIMENTAL RESULTS

Experiment 1. *Short periods of light or darkness at different times of day.*

This is the basic experiment, whose results will be presented in some detail. Four plants, A, B, C, and D, were brought into the laboratory; they were given, at the times shown in Table I, 1-hour periods of strong light (6–12 in. from a curved bank of eight 80-watt daylight fluorescent lamps) or darkness. At the end of each 1-hour experimental period a strip was removed; all four strips from each plant came from the same leaf. Between the experimental periods the plants were allowed to remain in the normal fluctuating light of the laboratory.

There are therefore 2 treatments (light and darkness) and 2 times of day (morning and afternoon) replicated on 4 leaves (A, C, B, D). The leaves are in fact subdivided according to order of treatment: A and B received the morning, C and D the afternoon, treatment first: in A and C light preceded darkness, in B and D darkness preceded light. These differences have not been taken out in the analysis of variance, but their effects will be briefly noted in due course. It must also be noted that the air temperature at the leaves of the plants in light (27° C.) was appreciably higher than that in darkness (18° C.), so that light is confounded with temperature. However, the results of Expt. 2 below suggest that the effects of such a temperature difference will be small. The results of Expt. 1, condensed into a table of means, have been incorporated in Table I; they show an increase in starch and a decrease in aperture both (i) in darkness as compared with light, and (ii) in the afternoon as compared with the morning.

TABLE I

Results of Expt. 1

(a) *Experimental arrangement*

Date	Time	Plant			
		A	B	C	D
18 May	09.00–10.00	Light	Dark	—	—
	10.00–11.00	Dark	Light	—	—
	15.00–16.00	Light	Dark	Light	Dark
	16.00–17.00	Dark	Light	Dark	Light
19 May	09.00–10.00	—	—	Light	Dark
	10.00–11.00	—	—	Dark	Light

(b) *Summary of means*

(Mean values for starch and aperture per stoma, in μ^2)

	Treatment		Time		Plant			
	Light	Dark	a.m.	p.m.	A	B	C	D
Starch	119·8	130·1	118·4	131·5	106·0	108·2	145·3	140·2
Aperture	64·1	48·8	62·3	50·5	49·4	57·1	53·0	66·1

TABLE II

Analysis of variance—results of Expt. 1
(*carried out on strip totals*)

Source of variance	D.F.	Mean square: starch	Mean square: aperture	Correlation coefficient
Within strips	304	1,470	642	+0·5053
Between strips	15	9,803	4,902	+0·0735
leaves	3	34,348	4,142	(+0·4513)
light	1	8,364	18,819	—
time	1	13,624	11,234	—
total interactions	10	2,202	3,105	(+0·6276)
leaf-light	3	794	1,757	—
leaf-time	3	2,889	3,993	—
time-light	1	379	616	—
2nd order	3	3,530	4,394	—

Analysis of results (*Expt.* 1)

(i) *Starch.* The analysis of variance is given in Table II. It is clear that 1st-order interactions are small compared with the residual 2nd-order interaction, so that all interactions can be pooled as error. The total interactions are not significantly greater than the error *within* strips; but it does not follow that these too can be pooled. It is probable that stomata from the same strip, and therefore closer together, will resemble each other more closely than will stomata from different strips; and since treatments are always referable to different strips, it is clear that greater reliance is to be placed on an estimate of error between, rather than within, strips. Taking therefore our 'total interactions' term as error, we find that the variance due to leaves is highly significant; that due to time of day is significant; that due to light is not significant. Two points must be made:

(*a*) Had we chosen 'variance within' as our error term, both leaves and time would have been highly significant, and light would have been significant. There is, therefore, a *suggestion* that there may indeed be a light effect on starch; but the verdict must remain 'not proven'. If it exists, it is clearly the smallest of the effects here studied, and it could only be substantiated by an experiment on a larger scale. A possible explanation of this equivocal result will be given later (Expt. 7).

(*b*) Examination of the results for individual leaves shows that the high variability lies almost entirely between the pairs (A, B) (C, D). Whether this is truly an effect due to order of treatment, or to an unfortunate selection of two pairs of plants of slightly different history and behaviour, it is impossible to say.

(ii) *Aperture.* Here again 1st-order interactions are small compared with the residual 2nd-order interaction, and all interactions can be pooled; but the resulting term is significantly greater than the variance within strips, so that there is in this case no alternative but to use the 'total interactions' term as

error. The variance due to light is then as expected significant; variances due to the other factors are not.

(iii) *Covariance.* Partial correlation coefficients are given in the last column of Table II. An interesting result is the significant *positive* correlation between starch and aperture within strips. This is almost certainly no more than a reflection of the variation in size between individual stomata; under any one set of conditions larger stomata have larger apertures and more starch. Correlation *between* strips is seen to be absolutely negligible. Further partition of this latter correlation is of dubious value, since in no case are the variances of the members of the pairs both significant, and in any case the correlations for light and time, being based on only two pairs of values, must of necessity be unity. However, it is perhaps interesting to note that the partial correlations thus obtained for 'between leaves' and 'total interactions' (the latter representing 'between strips' with main effects eliminated) are positive and of the same order as the correlation within strips, probably for the same reason: a leaf or a strip which happens on the whole to possess larger stomata will show correspondingly larger values for both starch and aperture.

(iv) *Preliminary conclusions.* The results strikingly confirm the tentative conclusions reached by Heath (1949) on qualitative observations; they show (*a*) a highly significant variation in mean starch-content of the stomata of different leaves, (*b*) a significant increase in starch in the afternoon as compared with the morning, (*c*) no convincingly significant change in starch-content as a result of short periods of darkness, (*d*) a significant decrease in aperture as a result of a short period of darkness, (*e*) no significant change in aperture for any other of the variables studied, (*f*) a positive correlation (*circa* + 0·5) between starch and aperture under any one set of conditions, almost certainly reflecting merely the difference in size of individual stomata, and (*g*) no evidence for any negative correlation between starch and aperture as a result of changes in illumination or in the time of day.

Experiment 2. Effect of temperature

Expt. 2 (a).

Since the change from light to darkness in Expt. 1 was accompanied by a change in temperature, Expts. 2 (*a*) and (*b*) were undertaken in order to ascertain whether or no this factor might have affected the results. Expt. 2 (*a*) was a preliminary test, carried out as follows: At 15.15 hours a plant was put into one of the dark chambers used in the previous experiment, at a temperature of 18° C.; at 16.15 hours a strip was taken in the usual way. The plant was then quickly transferred to an unlighted incubator at 27° C., and after a further hour a second strip taken from the same leaf. The two strips therefore represent a temperature difference similar to that existing between the light and dark conditions of Exp. 1, but in this case the leaf was in darkness throughout. Subsequent measurements on the strip showed a slight reduction in both starch and aperture, but a *t* test indicated that this change was not significant compared with the variation within the strips.

Expt. 2 (*b*).

This was a larger-scale experiment on similar lines. Three plants, E, F, and G, were at 08.00 hours put respectively into a refrigerator at 5° C., an incubator at 25° C., and an incubator at 37° C.; all were in darkness. After 3 hours a strip was taken from each plant; the plants were then interchanged. After a further 3 hours a second strip was taken and the plants interchanged again; after a further 3 hours the final strip was taken. All three strips from each plant came as usual from the same leaf. The experimental arrangement constitutes an unrandomized 3 × 3 Latin square, the three varying factors being (i) plants (E, F, G), (ii) temperature (5°–25°–37°), and (iii) time (11.00–14.00–17.00). Variations associated with (iii) could be due to either (*a*) a progressive change in starch as a result of continued exposure to darkness, or (*b*) a diurnal effect such as that exhibited in Expt. 1. These two factors are confounded; but since it was found that the variation associated with time was not progressive (the highest mean starch-content being found in the central period), it may reasonably be assumed that any such variation is likely to be due to a diurnal effect.

Analysis of results (*Expt.* 2 (*b*))

(i) *Starch.* The means are given in Table III. Analysis of variance has shown that here again the residual variance between strips is not significantly greater than that within strips; and again we have the problem of choosing the correct error term. If 'within strips' is taken as error, both temperature and time are highly significant, though the variation due to leaves is not significant in this case; if 'between strips' is taken, none of the variances is significant.

TABLE III

Expt. 2 (*b*)—*summary of means*

(Mean values for starch and aperture per stoma, in μ^2)

	Temperature			Time			Plant		
	5°	25°	37°	11.00	14.00	17.00	E	F	G
Starch .	151·2	164·4	142·7	141·8	167·8	148·7	151·6	145·6	161·1
Aperture	45·5	41·4	38·0	40·6	44·6	39·7	42·6	36·9	45·4

Admittedly the degrees of freedom available for error in a 3 × 3 Latin square are so few that only a very striking effect is likely to be significant; we may say, therefore, that although there is a suggestion of a temperature effect, manifest in this experiment as a maximum at 25° C., with a falling-off both at 5° C. and 37° C., the evidence is inconclusive. However, this effect, if it is real, will have acted in the opposite direction to the effect of light on starch recorded in Exp. 1, and the reality of that effect is thus rendered more probable. The suggestion of a diurnal effect is not inconsistent with the more striking effect exhibited (over a longer time-interval) in the results of Expt. 1.

(ii) *Aperture.* In this case the total variance between strips fails to reach significance compared with that within strips; we can therefore say with some confidence that this experiment provides no evidence whatsoever for any effect of temperature upon aperture over the range studied.

(iii) *Covariance.* It is interesting to note that the partial correlation coefficient within strips is positive (+ 0·5520) and remarkably close to that recorded for Expt. 1, no doubt for the same reason. The coefficient between strips is not significant, and is in any case of negligible value since the aperture variance is not itself significant.

Experiment 3. Diurnal rhythm under constant conditions

An elaborate factorial experiment was undertaken in order to investigate the existence of a diurnal rhythm under conditions of continuous light or darkness. Unfortunately the results were largely inconclusive, and I therefore do not propose to weary the reader with their detailed quantitative exposition. They are, however, of sufficient interest to warrant a brief qualitative account, and a note on the cause—so far as it can be ascertained—of the failure of the experiment may be useful to other workers in similar fields. The experimental arrangement comprised two 4 × 4 Latin squares, one in light and the other in darkness. The variables were (i) plants, (ii) time of day (05.00–11.00–17.00–23.00 hours), and (iii) successive strip number (i.e. whether the strip was the first, second, &c., taken from that leaf).

Results (Expt. 3)

The means for each of the four times are shown in graphical form in Fig. 2, in which the periods have been doubled to exhibit more clearly the form of the records. Considering aperture first, there is clearly no significant change in continuous darkness, but there is a striking rhythmic change in continuous light. This confirms the findings of Gregory and Pearse (1937), who used a continuously recording porometer. The minimum is in good agreement with that found (*circa* 02.00 hours) by them; the maximum is not in good agreement, but in view of the very different experimental conditions this is hardly surprising. The rhythms exhibited by starch are neither of them inconsistent with the result of Expt. 1. It would be unwise on these data to hazard any opinion as to whether the apparent displacement of the two curves (light and darkness) is or is not a real effect.

Of these results, statistical analysis shows that the aperture variance in light is significant; the starch variances are not. This is due, not to a small main-effect variance, but to an unprecedentedly large residual error, of a totally different order from that found for starch in previous experiments. The Latin-square arrangement precludes the identification of this error with certainty, but an examination of the results suggests that it lies in a high 1st-order interaction between time and strip-number. The effect does not seem to be progressive, so that it cannot be ascribed to the onset of 'dark rigor' or of

any similar phenomenom in light; but it is clear that in any future work along these lines the Latin-square arrangement should be abandoned in favour of complete replication, so that the interactions can themselves be studied.

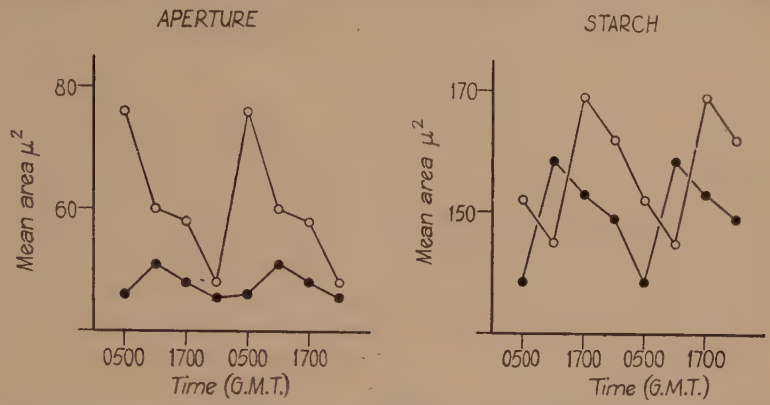

FIG. 2. Diurnal rhythm under constant light (open circles) or darkness (closed circles). (The first 4 points on each record are as measured, the second 4 are duplicates of these; see text, Expt. 3.)

Experiment 4. Enclosure in light

There is some evidence (Heath, 1949) that prolonged enclosure within a porometer-cup reduces the starch-content of the stomata; it is therefore to be expected that enclosure between glass plates in light will have a similar effect. This treatment, as is now familiar (Williams, 1949), causes the stomata to open widely as a result (Heath, 1950) of the diminished CO_2-content of the air so enclosed.

TABLE IV

Expt. 4—summary of means

(Mean values for starch and aperture per stoma, in μ^2)

	Treatment		Leaf	
	Enclosed	Not enclosed	A	B
Starch .	65·9	153·7	104·4	115·2
Aperture .	86·6	51·0	61·2	76·4

Expt. 4 is an extension of the preliminary experiment already reported by Williams and Spencer (1950). A plant was selected which bore two suitable leaves, practically opposite on the stem and equal in size. Part of each leaf was enclosed between two glass plates for $2\frac{1}{2}$ hours, the leaf being illuminated by a water-cooled 150-watt incandescent lamp. Then (at 15.00 hours) four strips were taken from the underside of each leaf, two from within and two from outside the glass plates. The strips were measured by the photographic technique, and the results are set out in Table IV. Enclosure has produced a mean decrease (51·1 per cent.) in starch and a mean increase (69·8 per cent.) in

aperture; analysis of variance has shown that the reduction in starch is highly significant, the increase in aperture is significant, and the difference between the leaves is not significant. The most striking feature of this result is the apparently high negative correlation between starch and aperture, conspicuous by its absence in the previous experiments; this phenomenon is investigated in the experiments which follow.

Experiment 5. Humidity in light

Heath (1949), in a brief discussion on the apparent reduction of starch in a porometer-cup, drew attention to the fact that enclosure in this manner does not leave the humidity unchanged; Expt. 5 is a somewhat untidy experiment designed to investigate the possible effect of this factor. Five plants (Q, R, S, T, U) were used, receiving the following treatments:

Plants Q and R

These were subjected to a modification of the 'glass plate' technique. The lower plate was, however, of $\frac{1}{2}$-in. 'Perspex' in which a circular depression, 1 in. in diameter and $\frac{1}{4}$-in. deep, had been drilled; this depression was filled with anhydrous calcium chloride to just below the rim. The area of leaf over the calcium chloride was thus enclosed but dry: the area between the plates outside this depression was enclosed but as usual rapidly became extremely wet. (A preliminary experiment using calcium carbonate in place of calcium chloride showed that there were no discernible effects due to, for example, optical differences caused by the introduction of a white substance into an otherwise transparent plate.) After 2 hours' illumination, at 12.00 hours, a strip was taken from each type of area. Q was illuminated by the usual fluorescent bank, but R (from considerations of space at the time) was illuminated by a water-cooled 150-watt incandescent lamp.

Plants S, T, and U

In each case two porometer-cups were fixed to the same leaf in the early afternoon, one of the cups being filled with anhydrous calcium chloride, the other containing a little water. Both cups were immediately darkened and allowed to remain overnight in this condition. The following morning the cups were illuminated with the 150-watt water-cooled incandescent lamp for 2 hours, during which time the porometer showed that the stomata opened rapidly; a strip was then taken from within each cup (S at 11.00, T at 12.00 hours). Plant U represents an early experiment carried out with the photographic technique: the time of stripping was not noted precisely, but is known to have been about midday.

Experimental arrangement

The unsatisfactory design of this experiment—due to a slight uncertainty of purpose at the beginning—will not affect the validity of the result, since the undesirable variations are all such as are likely to increase the error

variance. We may therefore regard it as comprising two factors with unequal replications: two techniques (glass plates, 2 replicates: cups, 3 replicates) and two treatments (wet and dry, 5 replicates each). Certain additional observations were made which have been excluded from this analysis, but which will be briefly considered in the sections which follow.

Results (*Expt.* 5)

(i) *Starch.* The results are given in Table V. First, there is a difference (which is highly significant) due to technique. This was expected; but since it probably arises as a result of the cumulative effect of a number of factors, I do not propose to discuss it further. Secondly, there is a difference (which is significant) due to treatment—an increase of starch under dry conditions. This interesting result—a direct effect of external humidity on the starch-content of the guard-cells—is not in fact a new discovery; it was reported by Iljin (1914), though this fact seems now to be largely forgotten.

TABLE V

Expt. 5— *summary of means*

(Mean values for starch and aperture per stoma, in μ^2)

	Technique		Treatment	
	Plates	Cups	Wet	Dry
Starch .	78·2	155·1	100·3	148·3
Aperture .	74·9	91·0	91·9	77·1

He states: 'Two specimens of *Campanula glomerata* with wide-open stomata gave a negative starch-reaction. After transference to a dry atmosphere the guard-cells . . . began to accumulate starch in considerable quantity.' He also allowed a shoot of *Origanum vulgare* to wilt in the light and noted that within half an hour a considerable quantity of starch had appeared in the guard-cells, though none had been detectable before. Finally, reverting to the present investigation, it is important to note for future reference (Expt. 7) that the interaction between technique and treatment was small and not significant.

(ii) *Aperture.* None of the changes in aperture is significant. It may be recalled that porometer measurements both by Heath (1950) and by Williams (1950) also failed to show any consistent difference in degree of opening between wet and dry cups. It seems clear, therefore, that in external humidity we have a factor by which we can bring about a change in starch-content without affecting the aperture.

Experiment 6 (*a*). *Humidity in light—comparison with unenclosed area*

It is clearly very important to ascertain whether the reduction in starch resulting from enclosure (Expt. 4) can be attributed solely to humidity. A comparison is needed between the wet and dry enclosed strips on the one hand and an unenclosed strip from the same leaf on the other. Such comparison

strips were in fact taken in the course of Expt. 5 in all cases except plant U and the results merit brief consideration; they are given in Table VI.

TABLE VI

Expts. 6 (*a*) *and* 6 (*b*)—*results* (*as strip totals, in* μ^2)

			Q	R	S	T
Expt. 6 (*a*)	Lower epidermis (starch)	wet	1,072	1,330	2,810	2,008
		dry	1,722	2,130	3,492	3,002
		outside	2,698	2,094	3,066	2,686
Expt. 6 (*b*)	Upper epidermis (starch)	wet	1,812	2,618	—	—
		dry	1,944	2,736	—	—
		outside	3,734	3,354	—	—
	Upper epidermis (aperture)	wet	1,440	1,787	—	—
		dry	1,760	2,020	—	—
		outside	962	1,064	—	—

Plants R, S, T (*incandescent light*)

In each of these three cases there has been a slight *increase* in starch as a result of dry enclosure; the increases are too small to warrant statistical examination, though it is interesting to recall that a similar effect had already been noted in a preliminary experiment (Williams and Spencer, 1950). This suggests that, for incandescent light at least, the reduction in starch can be attributed in its entirety to the change in humidity which accompanies straightforward enclosure.

Plant Q (*fluorescent light*)

This, the only leaf of this experiment under the fluorescent bank, has behaved rather differently; there is a reduction of starch-content in the dry area, less than that in the wet area but still well marked. Unfortunately, the conditions differ in so many respects—brighter light of a different spectral composition, a higher temperature—that discussion of the cause would be fruitless. Since there are in effect three replicates of the experiment in incandescent light, some reliance can be placed on that result; and the further analysis of the modification of the effect under fluorescent light must await further work.

Experiment 6 (*b*). *Humidity in light—upper epidermis*

In the 'plate' experiments above (plants Q and R), strips were also taken at the end of the experimental period from the upper epidermis in the appropriate regions, as nearly as possible immediately over the corresponding strips from the lower epidermis. Now, the dry conditions below the leaf would not be expected to have any appreciable effect on the upper stomata, separated as they are by the thickness of the leaf and therefore by a layer of presumably saturated mesophyll; and since the upper plate becomes noticeably wet, both enclosed strips should behave as 'enclosed, wet'. The results have been included in Table VI. The starch results are much as expected: a reduction in

both enclosed strips, greater in that (Q) under fluorescent light. However, the 'above dry' strips do show a slightly greater starch-content than their 'above wet' counterparts. The aperture results are surprising, since both the 'above dry' strips show a noticeable increase in aperture over their 'above wet' counterparts. The starch effect, if real, may reflect no more than a slight disturbance in the water-relations in that area; but if the aperture effect is real it suggests that the effect of exposing an area of the *lower* surface to a dry atmosphere is to induce an increased sensitivity to CO_2-deficiency in the stomata of the corresponding *upper* surface. This suggestion seems highly implausible, and would hardly be worth entertaining were there no other evidence. However, an increased sensitivity of the upper stomata to *increase* in CO_2-concentration in precisely these conditions has already been noted in passing by Heath (1950, p. 59); and the increase in transpiration from the intact lower surface of a *Sedum* leaf whose upper epidermis has been removed (Abou Raya, 1950) may perhaps be a cognate phenomenon.

Experiment 7. Humidity in darkness

This experiment has been carried out on two plants (V and W) with wet and dry porometer-cups attached exactly as for plants S and T in Expt. 5, except that the cups remained darkened until they were stripped at 11.00 hours. No strips from outside the cups were taken, as only the cups were darkened; and no 'plate' experiments were undertaken.

Results and analysis (Expt. 7)

The results are given in Table VII, and it is clear that there is this time no reduction in starch in the wet cup. The obvious method of handling these results is to combine them with those from the plants (S, T, U) which experienced similar treatment in light and to carry out a 2-factor (light and humidity) analysis of variance. When this is done, the interaction as expected

TABLE VII

Expt. 7—summary of means

(Mean values for starch and aperture per stoma, in μ^2)

	Plant V		Plant W	
	Wet	Dry	Wet	Dry
Starch .	191·7	181·8	185·9	164·6
Aperture .	54·1	71·8	82·1	93·4

greatly exceeds the main effects, but does not itself quite attain significance; the experiment is clearly on too small a scale. The results from the 'plate' experiments (plants Q and R) cannot be incorporated as they stand since this technique was not replicated in darkness; however, since it was found in Expt. 5 that the interaction between technique and treatment was negligible, it is permissible to use the *difference* between corresponding values for wet and dry conditions, whether in cups or between plates, as the variate. We then have

five such differences (plants Q, R, S, T, U) in light and two (plants V, W) in darkness; these differences are given in Table VIII.

TABLE VIII

Expts. 5 and 7—difference in starch area between dry and wet conditions (as difference between strip totals, in μ^2)

Condition	Plant	Difference (dry minus wet)
Light	Q	+ 650
	R	+ 800
	S	+ 682
	T	+ 994
	U	+1,672
Dark	V	−198
	W	−426

t (light-dark) = 3·97 (0·0 $> P >$ 0·01)

The difference in light and darkness is now significant; and we may therefore conclude that the interaction effect—a reduction in starch caused by the combined effects of light and high humidity—is real.

Implications of this result. Three points of interest arise:

1. Since these results refer to *enclosed* stomata, 'light' in fact means 'light and low CO_2', and 'darkness' means 'darkness and high CO_2'. Light and CO_2-concentration are irretrievably confounded and give us no guidance as to what might be the effect of light and high CO_2, or darkness and low CO_2; I hope in due course to undertake the experiments necessary to separate these effects.

2. This interaction phenomenon sheds considerable light on the results of previous workers. If humidity is high, an investigation of the effect of light on stomatal starch will elicit the classical reduction; if it is low there will be no effect. It is reasonable to postulate that at intermediate humidities the effect will be manifest to a greater or lesser degree. If the humidity effect is widespread (and in view of Iljin's results with *Campanula* and *Origanum* it may well be), this interaction would provide an elegant explanation of some at least of the contradictions in the literature, as well as of the typically equivocal results (such as that of Expt. 1 above) so often obtained.

3. In wet conditions, light produces a striking reduction in starch, and therefore (presumably) a striking increase in sugar: in dry conditions, light leaves the starch—and therefore presumably the sugar-content—unaffected: *but the aperture attained is the same in both cases.* The situation may be conveniently summarized in the following diagram:

	Starch		Aperture	
	Light	Darkness	Light	Darkness
Wet	Low	High	High	Low
Dry	High	High	High	Low

Comparison of the two 'light' columns shows that a change in humidity will bring about a change in starch but not in aperture: examination of the 'dry' row shows that a change from light to darkness will bring about a change in aperture but not in starch. In other words, by suitable manipulation of the external conditions *extensive changes in starch or aperture can be made independently of each other.*

Experiment 8. *Overall correlation*

On classical theory, if the mean values of starch and aperture for each strip are plotted against each other on a scatter diagram, in which each point represents one strip, some evidence of negative correlation is to be expected in

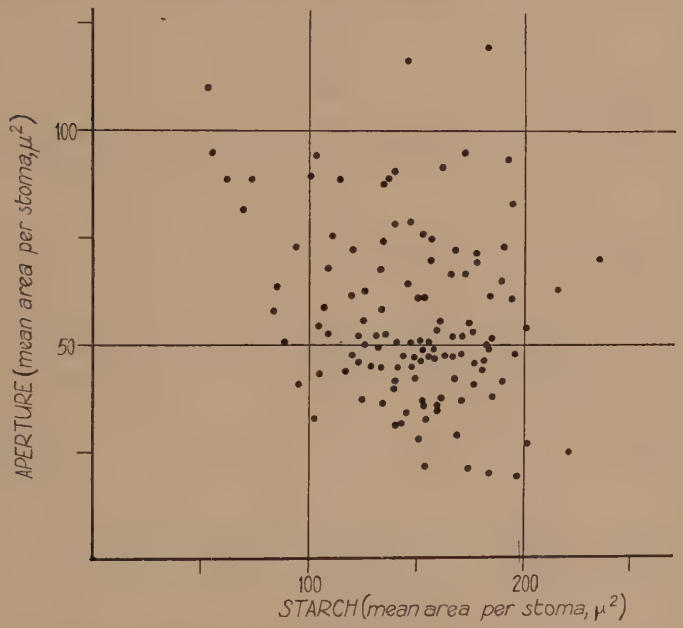

Fig. 3. Scatter diagram, starch-aperture: each point represents one strip (mean of 20 stomata). See text, Exp. 8.

spite of variation between plants. On the other hand, the conclusions of the last paragraph would lead us to expect no such correlation. Fig. 3 is such a diagram, in which have been entered the mean values for starch and aperture for every strip taken in the course not only of all the experiments described in this paper, but also of those observations on variation which will be reported separately. It will be observed that the points are so scattered that to calculate a correlation coefficient would be wasted labour; inspection alone is sufficient to show that the correlation is negligible.

There is, though, a further point of interest: one corner of the diagram is unfilled. *High aperture and low starch* is produced by light (and low CO_2) and high humidity: *high aperture and high starch* is produced by light (and low CO_2)

and low humidity: *low aperture and high starch* seems to arise largely as a result of individual variation in freely exposed leaves (which are exposed to light, relatively *high* CO_2 and relatively low humidity), and is as a matter of fact particularly characteristic of immature leaves: but no treatment here investigated produces *low aperture and low starch*. I venture to prophesy that when the projected interaction experiments are undertaken, the combination (light: high humidity: high CO_2) will fill the empty corner.

POSTSCRIPT

The function of the carbohydrate changes

We may assume, for there is some scattered evidence in its support (e.g. Sayre, 1926), that changes in starch-content are accompanied by inverse changes in sugar-content. However, it seems from the results here presented that these changes cannot be the *cause* of the turgor changes in the guard-cells; for were they so it is in the highest degree improbable that the great differences in starch-content observed as between wet and dry conditions in the light should not be accompanied by any discernible change in aperture. We may return with profit to an alternative hypothesis first advanced, so far as I am aware, by Kisselew (1925). For different reasons, he too came to the conclusion that starch-sugar changes could not be the cause of the turgor changes; he suggested that these carbohydrate changes might fulfil the useful function of reinforcing and stabilizing changes brought about by other means. It is most interesting in this connexion to recall that under excessively dry conditions, in which, although the aperture change in light is unaffected, the corresponding starch-sugar change is suppressed, both Williams (1950) and Heath (1950) have demonstrated that the stomata become unstable, exhibiting, once the master-factor controlling opening is withdrawn, an extraordinarily rapid closure. This, too, might be the cause of the well-known phenomenon that *Pelargonium* stomata react more rapidly to darkness in the late afternoon (when as a result of the inherent rhythm starch is high) than in the morning (when it is low); I know of no formal description of this curious property, but from Heath's (1949) remarks it is clear that he was familiar with it in 1939. It seems improbable that the starch-sugar change, undoubtedly widespread, should fulfil no function whatever, and Kisselew's hypothesis, which seems to fit the facts, is worthy of revival; but it must be confessed that it leaves the primary cause of the turgor changes even more mysterious than before.

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The Influence of the Plant Growth-regulator, 2-Methyl-4-Chlorophenoxyacetic Acid, on the Metabolism of Carbohydrate, Nitrogen and Minerals in *Solanum lycopersicum* (Tomato)

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SUMMARY

The effects of the foliar application of phytocidal concentrations of 2-methyl-4-chlorophenoxyacetic acid (MCPA) on change in total dry weight, and in 'available carbohydrate' (starch, 'total' and 'reducing' sugars), total nitrogen, phosphorus, potassium, calcium, and magnesium of 'tops' and roots of tomato plants have been followed over a period of 14 days following spraying.

There were two main treatments—'nutrient' (nutrient supply to roots continued after spraying) and 'water' (distilled water only supplied to roots after spraying)—the sub-treatments consisting of 'MCPA' versus 'no-MCPA' for each of the main treatments. Twelve different times of sampling were used.

In analysing the present data, the quantity 'residual dry weight' (total dry weight less 'available carbohydrate'), which was originally introduced by Mason and Maskell as a basis of reference for analyses of plant organs in short-period experiments not involving appreciable growth, has been used as an estimate of the permanent structure of plant growth. This new use of the 'residual dry weight' basis has brought out important features which were obscured when the data were left in their primary form (as percentages of total dry weight or amounts per plant).

Growth, as measured by increase in 'residual dry weight', was greatly inhibited by 2-methyl-4-chlorophenoxyacetic acid shortly after spraying, in both the presence and the absence of nutrient.

In the presence of 2-methyl-4-chlorophenoxyacetic acid, net assimilation rate (estimated as rate of increase in total dry weight per gram 'residual dry weight' of the 'tops') was greatly diminished while uptake of total nitrogen and of P_2O_5 (estimated as increase in total nitrogen or of P_2O_5 of the whole plant per day per 1 g. 'residual dry weight' of the roots) appeared to undergo a similar but much smaller diminution. It seemed probable, however, that in the presence of MCPA a larger proportion of the carbohydrate actually formed was utilized for synthesis of amino-acids and protein.

In the plant as a whole there was no evidence of actual depletion of 'available carbohydrate' as a result of MCPA treatment, this fraction showing a steady increase in all treatments throughout the experiment. The rate of increase was, however, much reduced by MCPA treatment. The 'tops' presented much the same picture as the whole plant, but for the roots the situation was quite different. While the roots of the 'no-MCPA' plants and also of the 'MCPA-water' plants showed a steady increase in available carbohydrate, those of the 'MCPA-nutrient' plants rose only very slightly (from the initial value of 8 mg. per plant to about 10 mg.) during the first 2 days, and then in the next 2 days declined to a value

(about 6 mg.) below the initial and remained at this low level for the rest of the experiment.

It is suggested that the phytocidal effect of 2-methyl-4-chlorophenoxyacetic acid in the presence of nutrient may be due to depletion of the 'available carbohydrate' supplies in the *roots*, which is shown to be brought about, in part, by reduced transport from the tops, and partly by the relatively greater utilization of the carbohydrate present. These results offer an explanation for the facts that plants showing vigorous growth are more easily killed by MCPA and that perennial plants, particularly those with storage tissues in their roots, are more resistant. Further, they suggest the useful practical application that MCPA treatment should be given when the carbohydrate reserves of the roots are at a minimum. For perennial plants, conditions might be expected to be optimal for the application of MCPA in late spring, at a time when the first 'flush' of growth is slowing down and before any appreciable new reserves of carbohydrate have been accumulated.

It was also shown that 2-methyl-4-chlorophenoxyacetic acid prevented the net synthesis of starch, but still permitted an appreciable net formation of sucrose.

2-methyl-4-chlorophenoxyacetic acid appeared to have no effect on the uptake of potassium, calcium, or of magnesium. The lack of effect on potassium is contrasted with the previous observation by Rhodes, Templeman, and Thruston (1950) that *sub-lethal* concentrations of MCPA, applied over a relatively long period to the *roots* of tomato plants, specifically depressed the uptake of potassium.

INTRODUCTION

In an effort to gain some clue to the mode of action of the phenoxyaceti acids, various investigators have determined the chemical changes concomi tant with the application of these substances to plants.

Mitchell and Brown (1945–6) working with *Ipomoea lacunosa* ('Annua Morning Glory') found that in plants to which 1,000 p.p.m. 2:4-dichloro phenoxyacetic acid in 0·6 per cent. carbowax 1500 had been applied as foliage spray, the sugar content first increased above that of untreated plants then decreased, while the starch-dextrin reserves were rapidly depleted. The concluded that 'readily available carbohydrates (sugars, starch, and dextrin were essentially depleted within a period of 3 weeks in plants that wer growing vigorously, and also in plants that were relatively dormant whe treated'.

Smith, Hamner, and Carlson (1947) working on *Convolvulus arvensis* (Bind weed) reported increases in total sugars of leaves, stems, and undergroun parts during the first few days after the foliar application of 2:4-D, followe by decreases to control levels. The starch-dextrins fraction decreased in a three, reaching one-third the control levels in leaves, roots, and rhizomes b the 10th day. Although these authors state that the changes in carbohydrat which they observed were 'in many ways similar to those observed b Mitchell', their figures give no indication of any reduction in 'availabl carbohydrate' as a result of 2:4-D treatment until at least 10 days after th application, by which time '10% to 20% of the leaves and stems were dead

They considered 'that the reduction in available carbohydrates whether by diminished production or increased utilization was not the principal cause of death'.

Rasmussen (1947) found that 2:4-D rapidly increased the reducing sugar content of dandelion roots and that the decrease in sucrose, dextrin, and levulin contents was considerably more than enough to account for the increase in reducing sugar. He concluded that 'the action on dandelion of 2:4-D in herbicidal concentrations is principally the destruction of carbohydrate reserves, with most of the loss being accounted for by increased respiration'. He believed, however, that these effects alone probably do not account for the lethal action of 2:4-D.

Sell, Luecke, Taylor, and Hamner (1949) reported that 1,000 p.p.m. 2:4-D applied to the foliage of Red Kidney Bean resulted in depletion of reducing and non-reducing sugars in the stems combined with 'a considerable reduction in carbohydrate reserves and a decrease in acid hydrolyzable polysaccharides'. Since they also found a large increase in protein in the stems of treated plants, they suggested that a large portion of the carbohydrates is utilized in protein synthesis.

Weller, Luecke, Hamner, and Sell (1950) in continuation of the above work showed that in the roots and leaves of the same plants, 2:4-D resulted in a depletion of non-reducing sugar, but no significant changes in starch or polysaccharide.

While 2:4-D obviously affects carbohydrate metabolism, it is clear from the above data that there is no general agreement as to the nature or significance of the changes involved.

With the publication of a method of starch determination by Pucher, Leavenworth, and Vickery (1948) which is specific for starch, it was considered that further useful data regarding the action of the phenoxyacetic acids on carbohydrates might be obtained by its use. In order to minimize changes in carbohydrate fractions during killing and drying which are apt to occur when the usual methods are employed, it was decided to employ lyophilic drying.

Since previous work by Rhodes, Templeman, and Thruston (1950) had shown that sub-lethal concentrations of 2-methyl-4-chlorophenoxyacetic acid (MCPA) applied to the roots of tomato plants over a fairly long period caused a marked diminution of the K_2O content of the 'tops' and a marked accumulation of potassium in the 'roots', the K_2O content of the plant as a whole being greatly lowered, it was also of interest to follow the changes in mineral and nitrogen content under the influence of foliar applications of phytocidal concentrations of MCPA.

Thus in the experiment which forms the subject of the present paper, data are presented for starch, total and reducing sugars, total nitrogen, P_2O_5, K_2O, CaO, and MgO. In order to enable the detection of any redistribution of minerals or nitrogen within the plant resulting from MCPA action, the masking effect of continued nutrient uptake was eliminated by supplying half

the plants with distilled water only after spraying, while the remainder continued to receive nutrients.

EXPERIMENTAL METHODS

Design of experiments. Three hundred and eighty-four uniform 'Stonor's M.P.' tomato (*Solanum lycopersicum*) plants, each about $7\frac{1}{2}$ in. in height, were available for experimentation on 17 May 1949. The plants were growing in an inert medium of flint sand contained in flower pots made of brown glass $4\frac{1}{2}$ in. in diameter supplied by the United Glass Bottle Manufacturers. The flint sand was supplied by Algernon Lewin Curtis of Chatteris (No. 4002 substitute) and gave an analysis of 0·0005 per cent. N and 0·62 milli-equivalents of replaceable bases per 100 g. sand. Nutrient solution was prepared as a concentrated solution which was diluted 100 times with tap-water before use. The concentrated solution contained the following commercial-grade salts:

Monammonium phosphate	437 g.
Magnesium sulphate (Epsom salts)	1,108 g.
Potassium nitrate	2,895 g.
Minor elements concentrate	4·5 litres
Sulphuric acid (AR conc.)	410 ml.
Tap-water to 45 litres	

On dilution 100 times with tap-water, this gave a nutrient solution at pH 6·0 containing 100 p.p.m. N, 60 p.p.m. P_2O_5, 300 p.p.m. K_2O, and 40 p.p.m. MgO.

Calcium was not added since an adequate amount was present in the tap-water.

The Minor Elements concentrate contained:

Boric acid, H_3BO_3	6·12 g.
Manganese sulphate, $MnSO_4,4H_2O$	9·75 g.
Ferric citrate, $FeC_6H_5O_7,3H_2O$	64·20 g.
Distilled water to 10 litres.	

Nutrient solution was supplied to the medium at intervals by a method of sub-irrigation in the following manner:

Eight metal trays (4 ft.×4 ft.×9 in.) painted on the inside with Mullard's Asphaltum Paint were arranged in a rectangle measuring 8 ft.×16 ft. on staging down the middle of a heated greenhouse. Each tray contained 48 of the potted tomato plants in a 7×7 arrangement, except for one row which had only 6 plants. The trays were connected, in two groups of four, to two nutrient storage tanks beneath the staging.

By means of centrifugal pumps attached to each storage tank, nutrient could be supplied at will to the experimental trays, flooding the potted tomato plants to any required depth. Usually nutrient was supplied twice daily, the liquid being allowed to rise just to the surface of the flint sand in the pots. When the pumps were stopped the liquid drained back into the storage tanks, leaving the trays empty once more.

The purpose of the experiment was to follow the changes in the amounts and distribution of certain plant mineral and carbohydrate constituents following upon the foliar application of 2-methyl-4-chlorophenoxyacetic acid. For soil-grown plants this is a difficult problem since the matter is complicated by the continued uptake of nutrients after the application of the growth-regulator, which may itself influence this process. By growing the plants in soilless culture, as in the present experiment, some of the plants can be supplied with water only, after the application of MCPA, while others continue to receive nutrients. Thus if the analysis is known for the plants immediately prior to the application of MCPA, any subsequent analyses can be related to this initial state, thereby enabling the changes in uptake and distribution of mineral salts, resulting from the MCPA treatment, to be followed.

With the sub-irrigation equipment described above, it was not possible to supply nutrient or water subsequent to MCPA treatment completely at random: a 'split-plot' design was therefore adopted.

The experimental design consisted of two main treatments, 'nutrient' and 'water', each being split into twenty-four sub-treatments for a comparison of 'MCPA' versus 'no-MCPA' at twelve different times of sampling. Two replicate blocks of all the forty-eight treatment combinations were arrayed as described below.

Owing to the fact that the glasshouse was orientated north and south and was completely shaded from the east, it was considered that any gradient in light intensity would be in the north/south axis. The replicate Blocks I and II were therefore arranged on the east and west sides of the house respectively in the most northerly compartment of a 3-section glasshouse, and the positions of the 'nutrient' and 'water' treatments were reversed in the two blocks.

Block I consisted of four trays A, B, C, and D, of which A and B provided the 'nutrient' treatment while C and D gave the 'water' treatment. Each tray contained 48 pots, providing 2 pots for every 'nutrient' sub-treatment in each of the trays A and B, and 2 pots for every 'water' sub-treatment in each of the trays C and D. Block II consisted of the remaining four trays, E, F, G, and H, in which the pots were allotted to the 'nutrient' or 'water' sub-treatments in a similar manner.

In each tray the arrangement of the pots assigned to the different sub-treatments was a random one so as to minimize any systematic errors arising from position.

At 7 a.m. on 17 May, on which date the experiment began, the storage tank connected to the experimental trays C, D, E, and F was drained and refilled with distilled water. At 8 a.m. and again at 9.30 a.m. all the experimental trays were flooded—A, B, G, and H with nutrient solution, and C, D, E, and F with distilled water. The liquid level was permitted, on each occasion, to rise about 1 in. above the surface of the flint sand so as to remove, in the case of the 'water' trays, any salts deposited on the surface by the previous sub-irrigation with nutrient solution. Thereafter trays A, B, G, and H received

nutrient solution, while trays C, D, E, and F received distilled water at each sub-irrigation period.

At 12 noon the plants which were to receive MCPA treatment ('MCPA') were removed from the trays and arranged in a 16 ft.×4 ft. rectangle in the middle section of the house. A square piece of cardboard, cut in the form of a collar, was placed around the stem of each of these plants so as to cover the glass pot and the surface of the flint sand. These plants were sprayed at 1.30 p.m. with 1,332 ml. of a 2,000-p.p.m. solution of the sodium salt of 2-methyl-4-chlorophenoxyacetic acid, this rate being equivalent to 2 lb. MCPA/acre in 100 gals. water/acre, which is commonly used in agricultural practice for weed-killing purposes. The solution was sprayed from a fine nozzle so that there was very little 'run-off'.

The plants remaining in the trays ('no-MCPA') were sprayed at 2 p.m. with an equivalent amount of water only. The 'MCPA' plants were then replaced, with their cardboard collars attached, in their randomized positions in the trays, the MCPA spray solution having had time to dry.

The initial sample was taken at 2.15 p.m., within ¾ hr. of beginning the MCPA application. Subsequent samples were taken at the following intervals:

Sample number	Time of sampling	Time after taking initial sample	Interval between sampling times
1 (Initial)	2.15 p.m. 17 May	0	—
2	10 p.m. ,,	8 hrs.	8 hrs.
3	6 a.m. 18 May	16 ,,	8 ,,
4	2 p.m. ,,	24 ,,	8 ,,
5	10 p.m. ,,	32 ,,	8 ,,
6	2 p.m. 19 May	2 days	16 ,,
7	2 p.m. 20 ,,	3 ,,	24 ,,
8	2 p.m. 21 ,,	4 ,,	24 ,,
9	2 p.m. 23 ,,	6 ,,	2 days
10	3 p.m. 25 ,,	8 ,,	2 ,,
11	2.30 p.m. 27 ,,	10 ,,	2 ,,
12	2.15 p.m. 31 ,,	14 ,,	4 ,,

At 7.15 a.m. on 18 May, 17 hours after taking the initial sample, the storage tanks were drained and refilled with nutrient and distilled water respectively, thus helping to free the 'water' treatment from any nutrients which may have been leached from the flint medium. This procedure was repeated on 21 May (4 days after spraying) for the distilled water storage tank.

At each sampling time sixteen 'MCPA' and sixteen 'no-MCPA' plants were removed, two of each category from every tray, according to the predetermined, randomized plan of selection. The stems of the four plants of a given sub-treatment from Block I were then cut at the insertion of the cotyledons. The four roots were washed free from flint sand, blotted to remove surface moisture, quickly cut into portions about 1 in. in length, and bulked in a tared flask suitable for use on the lyophilic drying apparatus. For the 'tops', however, two out of the four were discarded at each sampling time owing to the fact that the drying apparatus could not cope with the full

weight of 'tops'. The 'tops' to be discarded at each sampling time were decided by random selection before the experiment began. The remaining two 'tops' were quickly cut into coarse pieces and placed in separate tared flasks to be bulked after lyophilic drying had been completed. The corresponding plants from Block II were treated in similar fashion. All the flasks containing the plant tissues were then stoppered and immersed in a mixture of alcohol and 'Drikold' for 1 minute in order to freeze the tissues. The samples were held in 'Drikold' until they underwent lyophilic drying. The dry weights were determined and then the tissues were ground in a Christie Norris 5-in. mill yielding a very light, fine, green powder. In this manner, from each of the two blocks, four different kinds of bulked sample were obtained at each sampling time—'MCPA-nutrient', 'MCPA-water', 'no-MCPA-nutrient', 'no-MCPA-water'.

The samples were stored in desiccators in the dark and subsequently determinations were made of starch, 'total sugars', 'reducing sugars', total nitrogen, phosphorus, potassium, calcium, and magnesium, all as percentages of dry weight.

Methods for lyophilic drying and analysis

1. *Lyophilic drying.* An apparatus was used in which twenty-four tared 100-ml. flasks containing the plant samples were accommodated around two large spherical condensing surfaces.

The samples were left on the lyophilic drying apparatus for 15 hours approximately, followed by 24 or more hours in desiccators under high vacuum with activated alumina as desiccant. The flasks were then reweighed and the dry weights determined by difference.

A number of estimations for moisture content made by the Karl Fischer method on the stored ground samples which had been stored for several weeks showed that this was of the order of 3–5 per cent. Thus it was considered unnecessary to make corrections for moisture content in the subsequent analyses.

A few samples were examined for moisture content immediately after freeze-drying and desiccation. These were found to contain less than 2 per cent. moisture, showing that the drying method was satisfactory.

2. *Starch.* The method of Pucher, Leavenworth, and Vickery (1948) was adopted with minor modifications. The use of a boiling water-bath for hydrolysis of the extracted starch proved unsatisfactory, resulting in incomplete hydrolysis. Substitution of a glycerol bath at 105° C. gave complete recovery.

The factor of 0·129 used by Pucher, Leavenworth, and Vickery to convert ml. 0·005 N thiosulphate solution to mg. glucose proved too low under our conditions. Tests with pure, dry samples of a number of plant starches showed that a factor of 0·135 was necessary to give the theoretical yield of starch; this factor was therefore adopted in the analysis of our experimental material.

The figures for starch were determined from a single extraction, precipitation, and hydrolysis of each sample, followed by duplicate sugar estimations on the hydrolysate. The starch was reported as mg. starch.

3. *Reducing and total sugars.* Approximately 200 mg. of the dried material was extracted for 3 hours in a Soxhlet apparatus containing 80 per cent. alcohol, to which a small amount of AR Barium carbonate powder was added to prevent partial hydrolysis of 'non-reducing sugars' under the influence of heat and the acidity which would otherwise be developed by the presence of the tissues.

Neutral lead acetate was used for clearing followed by sodium oxalate in the usual manner. 'Reducing sugars' were determined using the Somogyi phosphate sugar reagent in the manner described by Pucher, Leavenworth, and Vickery (1948). An experimentally determined factor of 0·135 was used to convert ml. 0·005 N thiosulphate to mg. glucose.

'Total sugars' were determined by acid hydrolysis (5 ml. conc. HCl (s.g. 1·178) added to 75 ml. of solution) for 10 minutes at 70° C., of an aliquot of the lead-free filtrate prepared from the original dried material, followed by neutralization in the usual manner, and the estimation of the reducing value of the hydrolysate as described for 'reducing sugars'.

Single extractions of the plant material were followed by 'reducing sugar' determination in duplicate. Similarly, single hydrolysis of the 'non-reducing' sugars was followed by duplicate estimations of the resultant reducing value.

'Reducing sugars' were reported as mg. glucose, while 'total sugars' were reported as 'mg. invert sugar'.

A number of samples were analysed before and after storage for several months, from which it was clear that the storage conditions had not influenced the amounts of 'reducing' and 'non-reducing' sugars present in the samples.

4. *Potassium, calcium, and magnesium.* Calcium and magnesium were determined by the chemical methods described by Rhodes, Templeman, and Thruston (1950). Potassium was also determined by a chemical method found in the same paper, except that a modification proposed by Chapman (1947) was incorporated.

5. *Total nitrogen* was determined by a semi-micro Kjeldahl digestion followed by semi-micro distillation.

6. *Phosphorus* was estimated by the chemical method described by Rhodes, Templeman, and Thruston (1950).

EXPERIMENTAL RESULTS

(*a*) *Change in appearance of plants*

The following visual observations were made at intervals after the MCPA treatments had been applied:

2.15 p.m., 17 May. Initial sample taken. All plants looked uniform.
3.15 p.m., 17 May. Slight epinasty in MCPA plants.
10 p.m., 17 May. Very marked epinasty in MCPA plants.

2 p.m., 19 May. Very marked epinasty in MCPA plants. Stems swollen at a point slightly above the insertion of the first leaves. No morphological changes in the roots.

2 p.m., 20 May (3 days after spraying). Foliage of all 'water' plants distinctly paler than that of 'nutrient' plants, with cotyledons completely chlorotic and first pair of leaves yellowish.

Stems of MCPA plants swollen throughout their length. In MCPA-nutrient plants cotyledons and first pair of leaves were yellowish.

2 p.m., 23 May (6 days after spraying). 'Water' plants considerably smaller and paler than 'nutrient' plants, with cotyledons and first pair of leaves quite yellow.

First truss just visible on no-MCPA plants but not showing on remainder.

Stems of MCPA plants much contorted, outer layers of tissue split in several places, this effect being much more severe in the presence of nutrient. In MCPA-nutrient plants the first pair of leaves and the cotyledons yellow, but rest of foliage deep green.

MCPA plants showed a characteristic necrosis of leaf margins of the three leaves distal to the first pair; this effect became less severe as the stem was ascended.

3 p.m., 31 May (14 days after spraying). No-MCPA-nutrient plants 18 in. tall, the first truss beginning to flower with the second just visible. Five axillary shoots present, the lowest being about 4 in. in length.

MCPA-nutrient plants had very brittle stems, which were badly split, the outer layers sloughing off, revealing roots emerging beneath. Characteristic necrosis of leaf margins still present. Five swollen and dwarfed axillary shoots present.

No-MCPA-water plants had the first truss in flower. Foliage pale green with lower leaves yellowing.

MCPA-water plants had only two much-swollen and stunted axillary shoots. Stems very brittle. Characteristic necrosis of leaf margins still present, but foliage on lower half of the plants yellow or yellowing.

(*b*) *Changes in total dry weight and in components of dry weight*

The different treatments produced, as time went on, greatly increasing differences in dry weight so that the effects of treatment could not be readily judged from inspection of the analytical figures for the different components as percentages of the dry weight. The full analytical data have been deposited at the Natural History Museum and in Table I only the treatment means for dry weight, starch, 'total sugars', and 'reducing sugars' are presented.

To bring out the effects of the treatments the analytical data have been recalculated in various ways and the results are shown in Tables II–VI and Figs. 1–9. The results are dealt with under the following headings:

1. Dry weights and residual dry weights.
2. Rates of increase of different components per gram residual dry weight.

Table I

Changes in dry weight, starch, total sugars, and reducing sugars at intervals after treatment. (Means of two replicates)

Sample No.[1]	Time after spraying	Dry wt. (total of 4 tops or roots in g.)				Starch as % dry wt.				Total sugars (as invert sugar) as % dry wt.				Reducing sugars (as glucose) as % dry wt.			
		No-MCPA		MCPA		No-MCPA		MCPA		No-MCPA		MCPA		No-MCPA		MCPA	
		Nutrient	Water	Nutrient	Water	Nutrient	Water	Nutrient	Water	Nutrient	Water	Nutrient	Water	Nutrient	Water	Nutrient	Water
'Tops'																	
1	0 hrs.	4·83	4·54	5·25	4·51	7·4	6·7	8·2	7·5	3·2	3·8	4·3	4·2	2·2	2·7	2·8	2·9
2	8 ,,	5·15	5·25	5·04	5·13	7·4	8·8	7·4	9·7	2·0	2·8	2·6	2·8	1·2	1·6	1·8	2·0
3	16 ,,	4·80	4·73	5·45	4·21	5·1	7·6	4·2	5·3	2·2	2·6	2·9	3·4	1·4	1·8	2·1	2·6
4	24 ,,	7·05	5·29	6·21	5·17	8·2	10·8	4·8	7·7	4·0	5·1	3·7	5·8	3·2	4·2	3·0	4·9
5	32 ,,	6·27	5·84	6·32	5·53	7·8	14·0	5·6	4·6	2·8	5·0	4·8	4·9	2·1	3·6	3·9	3·8
6	2 days	8·39	7·52	7·06	6·53	9·0	14·2	5·0	8·9	5·0	6·8	5·1	9·0	4·4	6·3	4·8	8·4
7	3 ,,	9·15	8·76	8·17	6·68	4·6	10·1	0·8	5·0	3·6	7·6	5·2	10·9	3·6	7·2	5·2	10·2
8	4 ,,	9·34	9·50	8·24	6·11	9·0	15·7	2·1	6·6	5·4	9·0	6·8	12·4	5·3	8·6	7·0	12·5
9	6 ,,	12·66	10·61	10·88	9·27	7·0	16·7	1·0	6·1	8·6	10·6	8·4	17·3	6·8	9·0	7·2	15·3
10	8 ,,	18·11	12·20	11·61	8·21	8·8	18·4	1·3	5·7	8·0	10·8	5·9	17·8	5·4	8·5	3·9	15·2
11	10 ,,	20·38	13·77	12·88	9·11	11·0	27·1	1·1	4·8	8·6	11·7	7·2	21·8	6·1	9·2	5·1	19·1
12	14 ,,	33·82	18·65	17·21	11·24	11·6	28·0	1·7	3·0	10·2	11·6	9·0	23·6	6·6	8·8	5·8	20·2
Roots																	
1	0 hrs.	1·29	1·16	1·30	1·20	0·2	0·1	0·1	0·2	2·4	2·8	2·4	2·2	1·0	1·5	1·6	1·5
2	8 ,,	1·41	1·42	1·37	1·32	0·1	0·2	0·2	0·2	3·6	4·9	2·8	4·0	1·3	2·0	1·0	1·6
3	16 ,,	1·43	1·40	1·42	1·28	0·1	Nil	0·2	Nil	2·8	4·6	2·0	2·9	1·2	2·4	0·8	1·6
4	24 ,,	1·57	1·47	1·48	1·27	Nil	0·4	0·1	0·1	4·0	5·6	2·7	2·9	1·7	2·8	1·3	2·0
5	32 ,,	1·56	1·41	1·36	1·42	0·2	0·5	0·1	0·2	3·9	5·8	2·4	4·7	2·6	3·2	1·7	3·9
6	2 days	1·91	1·71	1·63	1·40	0·2	0·7	0·1	0·2	3·4	5·6	2·6	4·6	3·0	5·0	2·0	4·1
7	3 ,,	2·07	1·82	1·58	1·46	0·4	1·1	0·2	0·2	3·2	6·3	1·6	4·6	2·8	5·8	1·4	4·2
8	4 ,,	2·21	2·06	1·52	1·39	0·2	1·0	0·1	0·1	3·0	8·2	1·3	5·0	2·8	7·4	1·4	4·7
9	6 ,,	2·56	2·15	1·63	1·54	0·4	1·5	Nil	Nil	4·8	9·8	1·6	7·2	2·8	7·4	1·0	5·8
10	8 ,,	3·11	2·51	1·66	1·60	1·0	2·4	Nil	0·3	4·4	11·7	1·3	9·6	1·6	8·2	0·6	6·4
11	10 ,,	3·47	2·63	1·76	1·63	1·0	3·2	0·1	0·4	4·5	13·2	1·6	11·6	1·8	9·4	0·8	8·6
12	14 ,,	4·31	3·20	1·88	1·56	1·4	4·1	0·3	0·2	4·8	17·5	1·0	12·8	1·6	12·0	0·6	10·0

[1] Dates of sampling are given on p. 134.

3. Carbohydrates.
4. Nitrogen and phosphorus.

1. *Dry weights and residual dry weights.* Since a large part of the dry weight in some treatments is made up of starch and 'total sugars', it is clear that a better measure of organic growth would be obtained if variations in these raw materials could be allowed for. To this end a procedure similar to that adopted by Mason and Maskell (1928) will be used. Mason and Maskell calculated the residual dry weight as the difference between the dry weight and the 'total available carbohydrate'. They introduced residual dry weight as a basis for calculation of results for mature tissues or leaves in which there was no appreciable growth over the relatively short periods of time involved in the experiments (up to 1 or 2 days only), while owing, for example, to diurnal carbohydrate fluctuation, there *was* change in dry weight. Mason and Maskell showed that the coefficient of variation of residual dry weight per leaf, in such cases, was much less than that of dry weight or of fresh weight per leaf, and was so small that without appreciable error, residual dry weight could be taken as constant. Hence change in any component of dry weight, when expressed as amount per mean residual dry weight of the organ, was equivalent to actual change per organ.

In the present experiment, however, there is growth; residual dry weight is increasing, so that it has not the same significance as a constant basis of reference. Moreover, the actual weight (per plant or portion of plant) of each component determined is calculable from the dry weight and the percentage of dry weight figures. Nevertheless, since residual dry weight does not include the carbohydrate materials which fluctuate between treatments and which may be regarded as raw materials for growth, residual dry weight may be used as a measure of the permanent structure, increase in which can be used as a good measure of organic growth. It will be seen that its use brings out features which do not come out as clearly from the dry weight figures or percentage of dry weight figures.

In calculating residual dry weight the available carbohydrate will be taken as the sum of starch plus total sugars. While this probably does not embody the full amount of readily available carbohydrate, it obviously represents a large weight of material which is liable to considerable fluctuations in response to conditions which do not necessarily produce corresponding changes in growth.

When the residual dry weights, calculated in the above manner from the data of the present experiment, were examined statistically, it was found that the sub-treatment variance calculated for each sampling time showed a significant regression on the corresponding mean residual dry weight. Two transformations—log and square root—of the results were therefore made: both were successful in reducing the heterogeneity of the variances.

As we are here interested in effects on growth rate, the logarithmic transformations are to be preferred, since change in the logarithm of the weight,

during any given time interval, will be proportional to the mean relative growth rate during the interval. An analysis of variance was accordingly carried out on the logarithms of the residual dry weights.

It was found that the overall effect of 'nutrient' and 'water' failed to reach a significant level, although the effects of MCPA and sampling time were highly significant. As the interaction of MCPA and sampling times and of nutrient *v.* water and sampling times were also highly significant, the regression of the logarithmic transformations of residual dry weight upon time was calculated for each of the four main treatment combinations: MCPA-nutrient, MCPA-water, no-MCPA-nutrient, and no-MCPA-water. In each case it was found that the linear regression was highly significant, but that the deviations from the regression line were significant for no-MCPA-nutrient and highly significant for no-MCPA-water.

Because of these significant deviations from linearity, quadratic regressions were calculated for each case. The quadratic regressions were highly significant for MCPA-nutrient, no-MCPA-nutrient, and no-MCPA-water and significant for MCPA-water. The deviations from curvilinear regression were not significant in any case.

The second-degree equations that fit the data most satisfactorily are as follows, with the standard errors of the regression coefficients shown in brackets:

MCPA-nutrient	$y = 0{\cdot}781 + 0{\cdot}01722\,x - 0{\cdot}00016\,x^2$	
	$(\pm 0{\cdot}00216)$	$(\pm 0{\cdot}00005)$
MCPA-water	$y = 0{\cdot}731 + 0{\cdot}01022\,x - 0{\cdot}00011\,x^2$	
	$(\pm 0{\cdot}00216)$	$(\pm 0{\cdot}00005)$
No-MCPA-nutrient	$y = 0{\cdot}768 + 0{\cdot}02373\,x - 0{\cdot}00017\,x^2$	
	$(\pm 0{\cdot}00216)$	$(\pm 0{\cdot}00005)$
No-MCPA-water	$y = 0{\cdot}737 + 0{\cdot}01807\,x - 0{\cdot}00022\,x^2$	
	$(\pm 0{\cdot}00216)$	$(\pm 0{\cdot}00005)$

where $y = \log_{10}$ (residual dry weight of four plants in grams) and

$x =$ time in units of 1/3 day.

However, in order to compare the growth rates in the different treatments a linear relationship is desirable. Since the linear regression is highly significantly greater than the deviations from regression in each case, we may validly fit straight regression lines, whose equations are:

MCPA-nutrient	$y = 0{\cdot}806 + 0{\cdot}01118\,x$	
	$(\pm 0{\cdot}00063)$	
MCPA-water	$y = 0{\cdot}749 + 0{\cdot}00596\,x$	
	$(\pm 0{\cdot}00063)$	

No-MCPA-nutrient $y = 0{\cdot}795 + 0{\cdot}01726\,x$ $(\pm 0{\cdot}00103)$

No-MCPA-water $y = 0{\cdot}772 + 0{\cdot}00967\,x$ $(\pm 0{\cdot}00118)$

where y and x are in the same units as before.

The significant differences for comparing the regression coefficients are:

(*a*)	Between those for MCPA-nutrient and MCPA-water . . .	0·00180
(*b*)	,, ,, No-MCPA-nutrient and MCPA-nutrient or MCPA-water	0·00204
(*c*)	,, ,, No-MCPA-water and MCPA-nutrient or MCPA-water	0·00216
(*d*)	,, ,, No-MCPA-water and No-MCPA-nutrient . .	0·00327

Thus, apart from the pair of coefficients for MCPA-nutrient and no-MCPA-water, the regression coefficients are significantly different. We may therefore conclude that both the main treatments, MCPA and absence of nutrient, depressed the rate of growth.

The magnitude of the observed growth effects may perhaps be more easily appreciated if the above linear regression coefficients are converted to the usual form of relative growth rates. Multiplying by $2{\cdot}3 \times 3 \times 100$ we obtain the following estimates (as mean percentage increases per day over the whole period following spraying): MCPA-nutrient 7·71, MCPA-water 4·11, no-MCPA-nutrient 11·91, no-MCPA-water 6·67. As is clear from the quadratic regressions, the relative growth rates are not constant but fall with time. This does not, however, invalidate the comparison of the average rates over the period in question. It will be seen that removal of nutrient reduces the relative growth rate to about 55 per cent. of the corresponding controls, while spraying with MCPA reduces the relative growth rate to about 65 per cent. of the corresponding controls. The actual growth increments show, of course, more severe depressions with the two treatments.

2. *Rates of increase of different components per gram residual dry weight.* In view of the sampling variation it is not profitable to calculate rates for each interval between sampling times. However, an estimate of the level and drift of the rates has been obtained by calculating rates for three approximately equal intervals, namely, 0–5 days, 5–9 days, and 9–14 days. This subdivision of the time has the advantage that except for the final value at 14 days, the values used for a given component at the beginning and end of each period can be the means of at least two samples. For day 0, of course, the mean of all four treatments is taken. For days 5 and 9 the means (for each treatment) of days 4 and 6 and days 8 and 10 respectively are taken. In the calculation of the rates the linear mean of the residual dry weight values at the beginning and end of each period is taken as the estimate of mean value during the period. Since the percentage increase in residual dry weight

during a period is never greater than 122 per cent., the linear mean will not be more than 5 per cent. in error even if increase is exponential.

Since the linear means of the residual dry weights are used in subsequent calculations, they are tabulated in Table II.

TABLE II

Linear means of 'residual dry weight'
(Each weight represents 4 'tops' or roots)

	MCPA		No-MCPA	
	Nutrient	Water	Nutrient	Water
'Tops'				
0–5 days	6·47	5·13	6·79	5·85
5–9 ,,	10·00	6·25	12·53	8·00
9–14 ,,	13·33	7·36	21·05	9·91
Roots				
0–5 days	1·40	1·32	1·77	1·57
5–9 ,,	1·62	1·41	2·70	2·04
9–14 ,,	1·78	1·40	3·58	2·35

This method of computation is used to obtain estimates of net assimilation rate and of uptake of nutrients.

i. *Estimate of net assimilation rate.* Net assimilation rate per cm.^{2} of leaf or per gram of leaf cannot be calculated, but an approximate estimate of assimilation rate can be obtained by calculating the rate of increase in total dry weight per gram residual dry weight of the *tops* as in Table III.

TABLE III

Estimate of net assimilation rate (mg. increase in total dry weight per day per 1 g. 'residual dry weight' of 'tops')

	MCPA		No-MCPA		MCPA as % of no-MCPA	
	Nutrient	Water	Nutrient	Water	Nutrient	Water
0–5 days	158	122	217	210	73	58
5–9 ,,	71	45	183	106	39	42
9–14 ,,	77	69	148	127	52	54

It thus appears that the net assimilation rate is diminished by about 50 per cent. in the presence of MCPA both with and without nutrient and that an appreciable diminution occurs even in the first period (of 5 days).

It is of further interest to determine what fraction of the net assimilation is utilized in growth in the different treatments. To obtain an estimate of this fraction we need to calculate the net increase in residual dry weight for each period as a percentage of the increase in total dry weight of the whole plant during the same time interval. This has been done in Table IV, from which it is seen that the fraction of the net assimilation which is utilized in growth is greater in the presence of MCPA than in its absence but is greatly diminished, as might be expected, in the absence of nutrient. The first of

TABLE IV

Total gain in 'residual dry weight' as percentage of gain in dry weight of whole plant

	MCPA		No-MCPA		MCPA as % of no-MCPA	
	Nutrient	Water	Nutrient	Water	Nutrient	Water
0–5 days	92·8	60·8	83·3	62·8	—	—
5–9 ,,	97·4	46·4	78·6	40·4	—	—
9–14 ,,	82·7	66·7	74·4	48·8	—	—
Mean (allowing for length of each period)	90·6	58·8	78·8	51·3	115·2%	114·7%

these points is examined further in a later section, but it is worth noting that this might, of course, apply to many other situations in which rate of assimilation was depressed.

ii. *Uptake of minerals.* An estimate of the rate of uptake of total nitrogen, phosphorus, potassium, calcium, and magnesium is obtained by calculating the increase of a given nutrient in the whole plant per day per 1 g. residual dry weight of the *roots*. These data are given in Table V, from which it seems

TABLE V

Estimates of rate of uptake of minerals (as mg. increase in a given mineral of the whole plant per day per 1 g. residual dry weight of the roots)

	MCPA-nutrient	No-MCPA-nutrient	MCPA as % of no-MCPA	
Total nitrogen				
0–5 days	30·1	23·5	128%	
5–9 ,,	17·5	22·6	77%	76%
9–14 ,,	16·0	21·4	75%	
P_2O_5				
0–5 days	13·9	10·9	128%	
5–9 ,,	6·4	10·4	62%	79%
9–14 ,,	8·8	9·4	93%	
K_2O				
0–5 days	34·2	22·8	150%	
5–9 ,,	28·5	29·1	98%	109%
9–14 ,,	9·2	7·9	117%	
CaO				
0–5 days	10·9	11·8	92%	
5–9 ,,	6·0	8·9	67%	103%
9–14 ,,	7·6	5·8	131%	
MgO				
0–5 days	8·4	11·2	75%	
5–9 ,,	9·2	6·2	148%	115%
9–14 ,,	12·5	14·0	89%	

that MCPA produces no marked effect on the rates of nutrient uptake, but there is evidence of some diminution of uptake of total nitrogen and of P_2O_5 after the first 5 days.

The percentage reduction in nutrient uptake in the presence of MCPA is, however, less than the depressant effect on the net assimilation rate and very much less than the drop in available carbohydrate of the roots (see later). This suggests that in the presence of MCPA the efficiency of utilization of energy of metabolites for mineral uptake is not much impaired. This is in marked contrast to the specific depression of potassium uptake resulting from the long-continued application of sub-lethal concentrations of MCPA to the roots of tomato plants (Rhodes *et al.*, 1950).

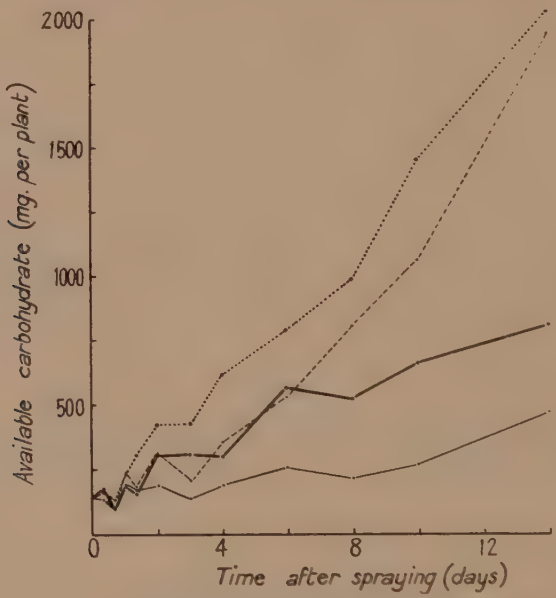

FIG. 1. Change in absolute weight of total available carbohydrate per plant with time.

MCPA+nutrient — Nutrient without MCPA
MCPA+water — Water without MCPA

3. *Carbohydrates.* In Fig. 1 the absolute weight of available carbohydrate present in the whole plant has been plotted. While, as might be expected from the lower residual dry weights, the MCPA plants contain less available carbohydrate than the corresponding no-MCPA plants, there is no indication that the carbohydrate reserves of the whole plant are 'essentially depleted' as a result of the action of MCPA, as was reported by Mitchell and Brown (1945–6) for *Ipomoea lacunosa* ('Annual Morning Glory'). On the contrary, the available carbohydrates show a slow but steady increase throughout the experiment, and at the end of 14 days, when the MCPA plants were approaching death, the MCPA-nutrient plants contained over 2·5 times and the MCPA-water plants over 6 times the initial amounts of available carbohydrate.

The greater amount of available carbohydrate per whole plant in the water as against the nutrient treatments must be due to a lower rate of utilization

of carbohydrate, since it is evident from Table III that rate of assimilation is depressed. The average depression is 20 per cent. and has a probability of less than 0·02.

Now when the 'tops' and roots are examined separately, a marked contrast is found. The 'tops' present essentially the same picture as the whole plant. On the other hand, while the roots of the no-MCPA plants and also of the MCPA-water plants show a steady increase in available carbohydrate, those

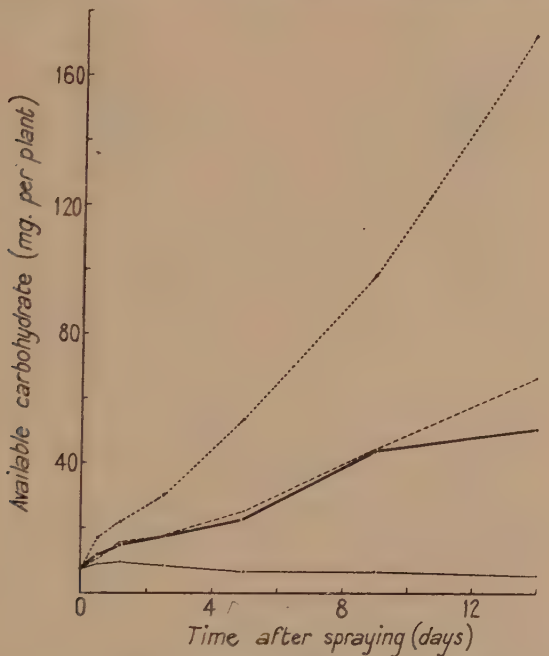

FIG. 2. Change in available carbohydrate per plant in roots with time.

MCPA + nutrient
MCPA + water
Nutrient without MCPA
Water without MCPA

of the MCPA-nutrient plants rise only very slightly from the initial value of about 8 mg. per plant to about 10 mg. during the first 2 days, and then in the next 2 days decline to a value below the initial and remain at this low level, about 6 mg., for the rest of the experiment (Fig. 2). This results in a marked decrease in the ratio of available carbohydrate in the roots to that in the whole plant for the MCPA-nutrient treatment and a similar but less marked effect for the MCPA-water treatment (Fig. 3).

It is therefore suggested that the phytocidal effect of MCPA in the presence of nutrient may be due to depletion of the available carbohydrate supplies in the *roots*. That this depletion is partly due to interference with translocation is shown from the data of Table VI, where gain in total dry weight by the roots (i.e. materials transported) is expressed as a percentage of the gain in

total dry weight by the whole plant (i.e. net synthesis). Thus the MCPA plants, both in the presence and absence of nutrient, transport only about 45 per cent. of the amounts transported in the corresponding no-MCPA plants. This drop in transport to the roots as a result of MCPA treatment is of interest since Eames (1950) has found that 2:4-D, applied to seedlings

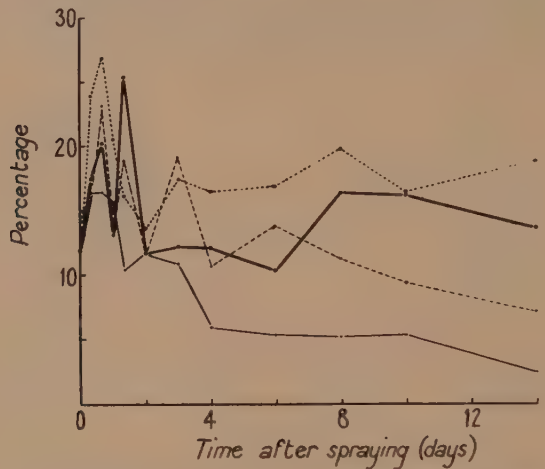

FIG. 3. Change with time in available carbohydrate of roots as percentage of available carbohydrate in whole plant.

MCPA+nutrient ----- Nutrient without MCPA
MCPA+water ..... Water without MCPA

TABLE VI

Gain in total dry weight by roots as percentage of gain in total dry weight by whole plant

	MCPA		No-MCPA		MCPA as % of no-MCPA	
Days after spraying	Nutrient	Water	Nutrient	Water	Nutrient	Water
0–5	6·6	7·3	15·6	14·2	—	—
5–9	4·6	13·4	9·8	13·6	—	—
9–14	3·3	−2·4	6·5	10·0	—	—
Mean gain in dry wt. by roots as % of gain in dry weight by whole plant (allowing for length of each period)	4·85%	5·58% (4·62)	10·69%	12·52%	45·3%	44·6%

of *Phaseolus vulgaris*, disrupts the sieve tubes and companion cells of the phloem in the stem and finally leads to the disappearance of all phloem tissue.

However, since this reduced transport to the roots does not prevent a steady rise throughout the experiment of available carbohydrate in the roots of the

MCPA-water plants, this must mean that the differential depletion of available carbohydrate in the MCPA-nutrient roots is due to the greater utilization of carbohydrate which has been shown to occur in the presence of nutrient and MCPA.

While the phytocidal effect of MCPA in the presence of nutrient may be due to depletion of available carbohydrate after the above manner, this

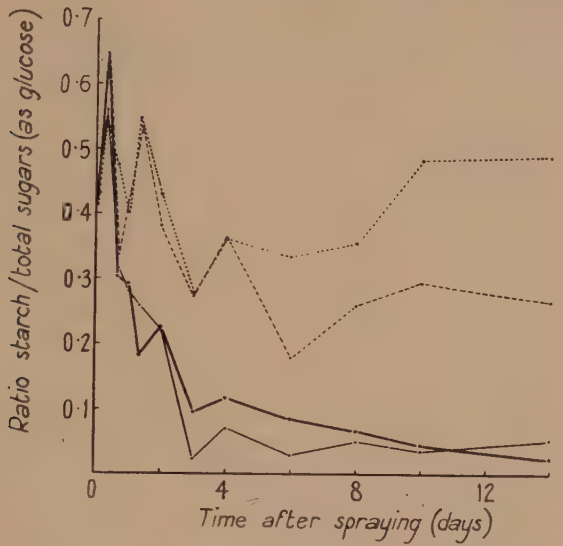

FIG. 4. Change in ratio $\frac{\text{starch}}{\text{total sugars (as glucose)}}$ with time.

MCPA+nutrient — Nutrient without MCPA
MCPA+water — Water without MCPA

obviously does not apply to the MCPA-water plants. The sickly state of these plants at the end of the experiment probably reflects mineral starvation rather than an effect of MCPA alone, since the corresponding no-MCPA plants showed similar symptoms.

These results offer an explanation for the facts that plants showing vigorous growth are more easily killed and that perennial plants, particularly those with storage tissues in their roots, are more resistant to MCPA. They might have useful practical applications since they suggest that MCPA is best applied when the carbohydrate reserves of the *roots* are at a minimum. For perennial plants, conditions might be expected to be optimal for the application of MCPA in late spring, at a time when the first 'flush' of growth is slowing down and before appreciable new reserves of carbohydrate have been accumulated.

It is of interest to study the carbohydrate changes a little more fully. In Fig. 4 the ratio of starch to total sugars (as glucose) for the whole plant has been plotted against time. It is clear that in both MCPA treatments there is a decrease in the ratio of starch to total sugars within 24 hours of spraying

and that this change becomes rapidly more pronounced with time. In the no-MCPA treatments, however, this ratio falls only slightly throughout the experiment.

The MCPA-induced change in the ratio of starch to total sugars may be due to changes in any of the following rates:

i. Increased rate of hydrolysis of starch.
ii. Lower rate of utilization of sugar.
iii. Decreased rate of synthesis of starch.
iv. Increased rate of breakdown of fats, reserve polysaccharides, &c., to give sugars.

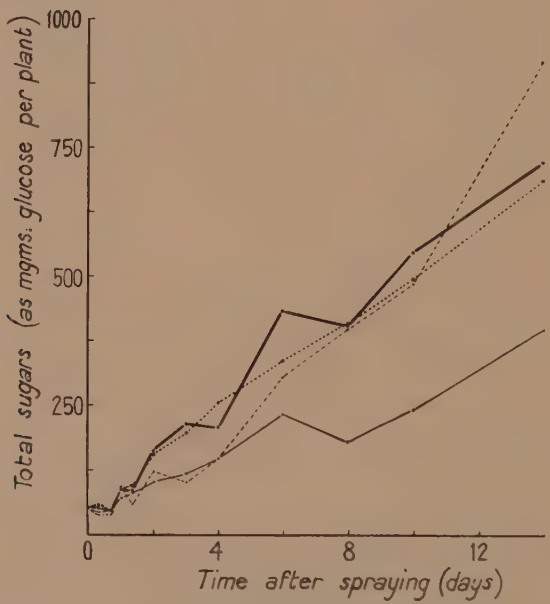

FIG. 5. Change in absolute weight of total sugars per plant with time.

MCPA + nutrient — Nutrient without MCPA
MCPA + water — Water without MCPA

By plotting the absolute weight of total sugars and of starch for the whole plants, as has been done in Figs. 5 and 6 respectively, it is possible to gain some information regarding these suggestions.

It is seen that, in general, total sugars increase with time in all treatments. This increase in total sugars in the MCPA treatments is much greater than can be accounted for even if all the initial starch were hydrolysed.

The much greater amount of sugars in the MCPA-water as opposed to the MCPA-nutrient, and the fact that the no-MCPA-water plants contain about the same amount of sugars as the corresponding nutrient plants in spite of their much lower residual dry weight, suggests that the water treatments seriously interfered with the utilization of sugars. This necessarily implies that sugar must continue to be utilized in the MCPA-nutrient plants.

Turning now to the curves for starch in Fig. 6 we find that in both water treatments greater amounts of starch are present than in the corresponding nutrient plants, even though the residual dry weights of the water plants are much less than in the nutrient treatments. This again points to interference with carbohydrate utilization in the absence of nutrient.

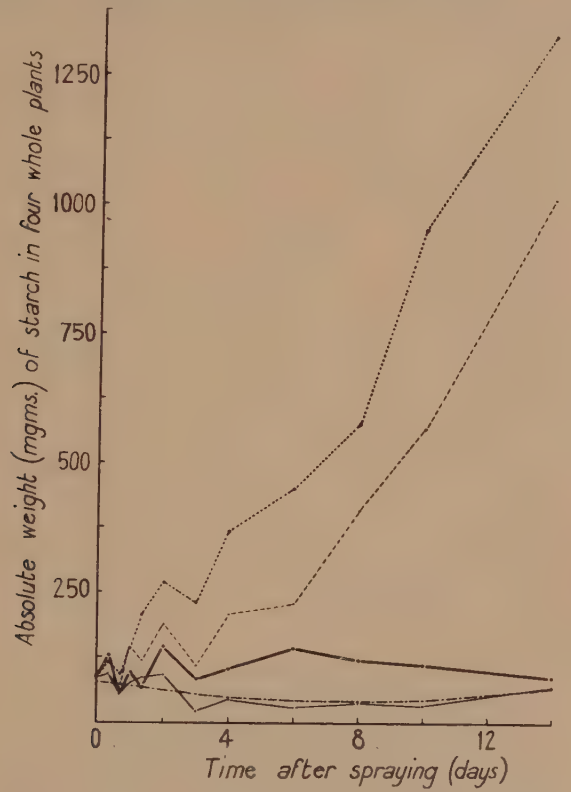

FIG. 6. Change in absolute weight of starch in 4 whole plants with time.

MCPA + nutrient
MCPA + water
Nutrient without MCPA
Water without MCPA
Fitted curve

There is a striking contrast between the amounts of starch present in the no-MCPA and MCPA plants. In the former the quantity of starch increases rapidly throughout the experiment, but in the latter the weights of starch do not rise above the initial amounts in spite of big increases in residual dry weight. Thus MCPA clearly prevents the net synthesis of starch. Moreover, an analysis of variance of the MCPA results, separately, showed that in the presence of nutrient there were significant differences between the amounts at different sampling times.

In the presence of nutrient there was no continued decrease in the net amount of starch from the time of spraying with MCPA to the end of the

experiment, but the results may be fitted by a second-degree curve (shown in the figure) which decreases to a minimum at 7·6 (± 2·0) days and returns almost to the initial value at 14 days. The second-degree constant (which produces the rise in the latter part of the curve) is significant at the 1 per cent. level.

The above analysis of the data therefore suggests that MCPA, in the presence of nutrient, induces the net hydrolysis of starch reserves soon after

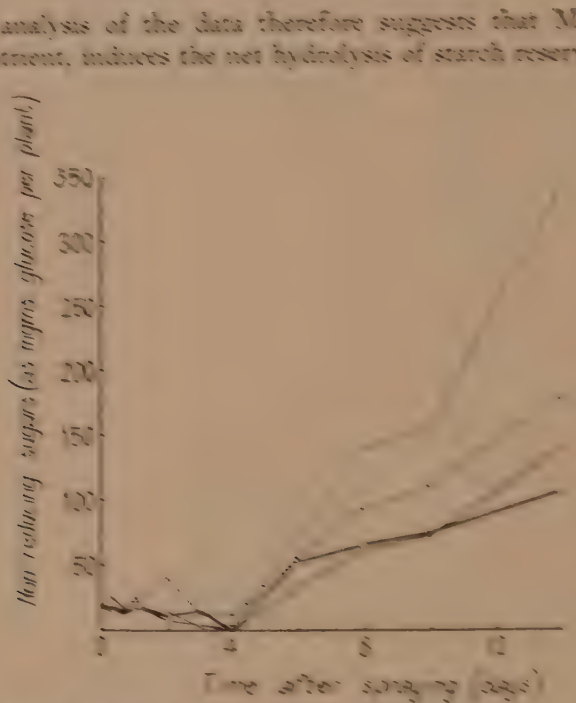

Fig. 7. Change in absolute weight of non-reducing sugars (as glucose) per plant with time.

MCPA + nutrients ——— Nutrients without MCPA
MCPA + water ——— Water without MCPA

spraying, but that this effect may show a partial reversal with time. It is clear, however, that, as in the MCPA-water treatment, the amount of starch present never rises above the initial value.

In view of the inhibition by MCPA of net synthesis of starch it is of interest to determine whether or not the net synthesis of disaccharides is also inhibited. For this purpose it can be assumed that calculation of non-reducing sugars as the difference between total and reducing sugars gives a measure of the amounts of sucrose present. These data are presented in Fig. 7.

It is seen that over the first 4 days of the experiment there is, in general, a slight fall in non-reducing sugars, followed by a fairly rapid rise for all treatments throughout the remainder of the experiment. It is obvious that quite an appreciable amount of sucrose is synthesized in spite of the MCPA treatments.

Little is known of the mechanism of sucrose synthesis in higher plants

but Hanes (1940) has demonstrated that phosphorylase is involved in the synthesis of starch in the potato. Hanes and Maskell (1942) have calculated from the equilibrium data for the reaction:

Starch + inorganic phosphate $\rightleftharpoons$ glucose-1-phosphate (ester-P)

that corresponding ionic species of the free and ester phosphates are, at

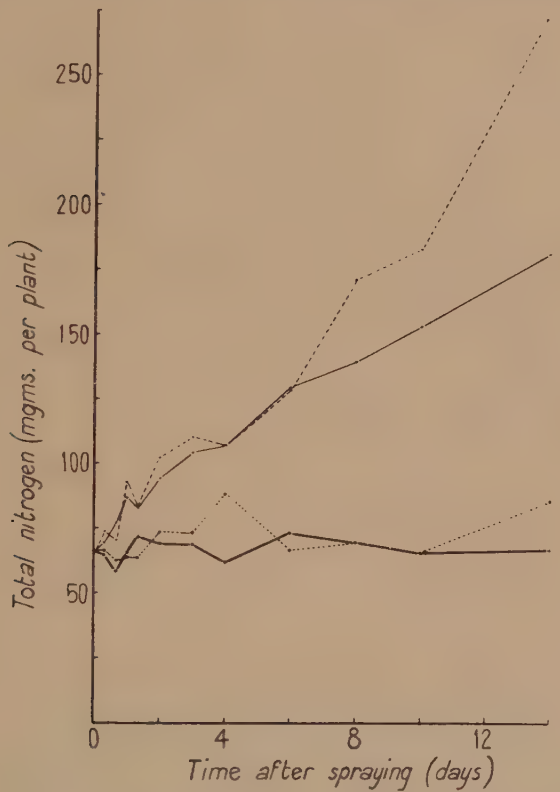

FIG. 8. Change in absolute weight of total N per plant with time.

MCPA + nutrient — Nutrient without MCPA
MCPA + water — Water without MCPA

equilibrium, present in constant ratios, and that the observed dependence of the equilibrium ratio,

$$\frac{\text{total glucose-1-phosphate}}{\text{total free phosphate}},$$

on pH is explicable on the basis of the known dissociation constants of the free phosphate and the ester phosphate. Since any change of pH would equally affect any glucose-1-phosphate and free phosphate involved in an equilibrium with sucrose and fructose, it appears unlikely that MCPA, which prevents net starch synthesis but permits sucrose synthesis, can be acting through this effect of pH on the dissociation of phosphates.

In view of the fact that sucrose synthesis takes place and since this presumably requires a phosphorylation of sugar, we cannot postulate an inhibition of phosphorylation. If, however, there is normally proceeding both synthesis of starch via phosphorylase and also direct or indirect hydrolytic breakdown of starch, then a possible explanation lies in a depression of starch phosphorylase activity relative to amylase or glucose phosphatase activity.

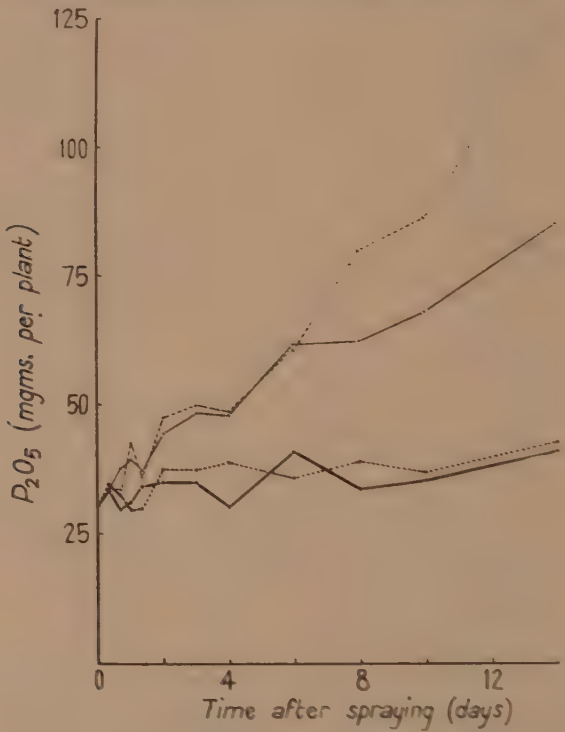

Fig. 9. Change in absolute weight of P_2O_5 per plant with time.

MCPA + nutrient — Nutrient without MCPA
MCPA + water — Water without MCPA

4. *Nitrogen and phosphorus.* In Figs. 8 and 9 the absolute weights of total N and of P_2O_5 respectively are shown against time. It is reasonable to assume that most of the N and P represents organically bound forms of these elements. It is at once apparent that the lower rate of carbohydrate utilization found in the water treatments, and which has been discussed above, may well be due to a failure of net amino-acid and protein synthesis owing to the absence of supplies of inorganic nitrogen and perhaps of phosphorus.

We have already noted (Table III) that net assimilation (estimated as rate of increase in total dry weight per gram residual dry weight of 'tops') was reduced to less than 50 per cent. of normal by MCPA treatment. Similarly we have seen (Table V) that uptake of total nitrogen and of P_2O_5 (estimated as rate of increase in total nitrogen or of total P_2O_5 per gramme residual dry

weight of the roots) also appears to be diminished, but to a much lesser extent, in the presence of MCPA. It would now seem probable that although production of carbohydrate and uptake of nitrogen and of phosphorus are lowered, a larger proportion of the carbohydrate actually formed is utilized for synthesis of amino-acids and protein in the presence of MCPA. This is to be expected since we have already seen (Table IV) that the fraction of the net assimilation which is utilized in growth is greater in the presence of MCPA than in its absence. The lower proportion, relative to residual dry weight, of available carbohydrate in the MCPA-treated plants would thus be due in part to lower assimilation rate, and partly to relatively increased utilization of carbohydrate, probably for amino-acid and protein synthesis. The same explanation would account for the observations made by Sell, Luecke, Taylor, and Hamner (1949), from which they concluded that under the influence of MCPA a large portion of the carbohydrates is utilized in protein synthesis. It is possible, however, that a reduction in net assimilation rate, combined with little change in rate of uptake of nitrogen and phosphorus, might result in increased carbohydrate utilization relative to the carbohydrate available. If the same carbohydrate, nitrogen, and phosphorus were present in both the MCPA and no-MCPA treatments, it appears doubtful whether MCPA would then result in any greater utilization of carbohydrate.

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I wish to acknowledge the liberal help received from a number of colleagues during the course of this work. My thanks are therefore offered to Messrs. P. A. Collier and W. D. Ewan for the experimental design and statistical analysis of the data, to Mr. M. N. Thruston for the design of the lyophilic-drying apparatus, to Messrs. Thruston and J. H. Dunn for the mineral and nitrogen analyses, to Mr. E. R. Armitage for help in the preliminary trials of the method of starch determination and for the routine analyses for starch, to Miss J. M. Blanchard and Miss S. M. Cooke for technical assistance, and to Dr. W. G. Templeman for his advice and interest throughout.

In developing and presenting the new use made in this paper of the 'residual dry weight' basis, I have had much helpful comment and advice from Dr. E. J. Maskell, F.R.S. The methods used to obtain from the data comparative estimates of rates of net assimilation and of uptake of nutrients were suggested by him, as were also the logarithmic transformations and the regressions on time used in the analysis of the growth data. I am particularly indebted to him for these suggestions and for his interest and constructive criticism on many other points during the preparation of this paper.

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The Metabolism of Glucose and Acetate in *Aspergillus niger*

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SUMMARY

1. The metabolism of a citric-acid-forming strain of *A. niger* when grown on a glucose-acetate medium has been investigated.
2. Acetate greatly accelerated the rate of utilization of glucose.
3. Citric acid production from glucose was much increased by the presence of acetate.
4. The formation of oxalate from glucose-acetate cultures was much less than from acetate alone.
5. In some cultures large amounts of glucose and acetate were consumed but no acidic products were formed.

INTRODUCTION

It has long been known that *Aspergillus niger* can convert glucose to gluconic, citric, and oxalic acids and can also produce the two latter acids from certain salts of acetic acid. It is now considered probable that the later stages of the conversion of glucose to citric acid are closely related to the tricarboxylic acid cycle first described by Krebs and Johnson (1937). Further, the processes by which acetate is converted by the mould to citrate, whether involving carboxylation, Thunberg condensation, or other means, are also probably connected with this cycle. Hence, if the metabolism of the two substrates follows a common path, the production of citrate from one should be affected by the other. The experiments described in this communication were undertaken to gain information on this subject.

EXPERIMENTAL METHODS

A. *Cultures*

All cultures were prepared from the strain N1 of *A. niger* which, when grown on a sugar medium, normally produces gluconic, citric, and oxalic acids, all in rather low yield, that for citric acid usually varying from about 8 to 15 per cent. on the glucose utilized. The basal medium employed (Currie, 1917) was of the composition:

NH_4NO_3: 0·200 g./100 ml.
KH_2PO_4: 0·075 g./100 ml.
$MgSO_4\ H_2O$: 0·021 g./100 ml.
HCl (11·3 N): 0·100 ml./100 ml.

The carbon sources (glucose and acetic acid) at the appropriate levels were incorporated before adjusting the pH with sodium hydroxide. The medium

was then filled into conical flasks (100 ml. or 500 ml.) in a volume corresponding to 40 per cent. of the capacity of the vessel. Sterilization was effected in free steam for three successive days. The medium was sown with 1 ml./50 ml. medium of a spore suspension containing *c.* 10^9 spores/ml. in 0·1 per cent. calsolene oil HS (I.C.I. Ltd.), prepared from slopes on glucose agar, which had been incubated for 10 days. All cultures were grown in the dark at 30° C.

B. *Analytical techniques*

Oxalic acid was determined by precipitation of the Ca salt and titration with permanganate (Halliwell, 1950). *Citric acid*: The pentabromoacetone method of Taussky and Shorr (1947) proved to be the most satisfactory of a number examined. *Acetic acid*: The acidified sample was saturated with $MgSO_4$ and distilled in steam in the Markham apparatus (1942) and the distillate titrated with alkali. *Glucose*: This was determined by the colorimetric ferricyanide method of Horvath and Knehr (1941).

EXPERIMENTAL RESULTS

Expt. 1. *Preliminary experiment on the metabolism of glucose at 8 per cent. and at 10 per cent. concentration in the presence of acetic acid at 2 per cent. concentration*

The medium was adjusted to pH 6·8 under the conditions shown in Table I. Here it is noted that the primary effect of acetate was strikingly to increase the consumption of glucose at both the 8 and 10 per cent. levels. The reciprocal effect was noticeable to a small extent on the fourth day, but later the higher concentration of glucose exerted a slight sparing effect on the acetate. In subsequent experiments it was found that 2 per cent. acetate was used even more rapidly than in the above cases, when 14 per cent. glucose was present.

The citrate yields were low throughout and the addition of acetate while raising the total yield did so only because of the enhanced combustion of nutrients. The yields on substrates consumed were markedly lower when acetate was added. Oxalate formation in the presence of acetate was increased from 5 to 20 times that obtained from glucose as sole substrate. Even so, the amounts formed from such glucose-acetate mixtures under conditions so favourable for formation of oxalate (higher pH) were less than from a culture grown solely on acetate (see below) at a comparable period of acetate consumption. It should further be observed that at the higher level of glucose in Table I less oxalate was produced than with 8 per cent. glucose even allowing for the slightly smaller acetate utilization.

Expt. 2. *The effect of different levels of acetate on the metabolism of glucose*

Further work was performed with initial pH values of 3·0–6·5. Growth occurred at all acidities in the absence of acetate, at pH 5·5, 6·0, and 6·5 with

TABLE I

Effect of acetate on the utilization of glucose

Treatment	Age (days)	g. carbon/100 ml. solution					Citrate carbon as % total C used	Acetate used as % initial acetate	Glucose used as % initial glucose	Total C used as % total initial C
		Glucose	Acetate initial	Total	Citrate	Oxalate				
A	0	3·28	0	3·28						
B		3·28	0·86	4·14						
D		4·20	0	4·20						
C		4·20	0·86	5·06						
		used			synthesized					
A	4	0·94	0	0·94	0·072	0·001	7·7	—	29	29
B		2·20	0·39	2·59	0·089	0·003	3·4	45	57	63
D		1·56	0	1·56	0·113	0·001	7·3	—	37	37
C		2·81	0·50	3·31	0·134	0·003	4·1	58	67	66
A	6	1·32	0	1·32	0·099	0·006	7·5	—	40	40
B		2·92	0·69	3·61	0·171	0·072	4·7	81	89	87
D		1·92	0	1·92	0·168	0·008	8·8	—	46	46
C		3·79	0·64	4·43	0·151	0·013	3·4	75	90	88
A	8	1·97	0	1·97	0·216	0·011	11·0	—	60	60
B		3·09	0·81	3·90	0·168	0·211	4·3	94	94	94
D		2·60	0	2·60	0·274	0·012	10·5	—	62	62
C		3·94	0·70	4·64	0·264	0·056	5·7	82	94	92

1 per cent. acetate, at pH 6·0 and 6·5 with 2 per cent. acetate, but only at pH 6·5 with 3 per cent. acetate (Table II).

The above results again indicated that 1 and 2 per cent. acetate considerably enhanced the rate of utilization of glucose at 8 and 14 per cent. concentrations. At the higher level of glucose (14 per cent.) acetate in 3 per cent. concentration was more uncertain in this effect. In a similar manner the yields of citrate were increased by the presence of 1 per cent. acetate although 2 and 3 per cent. acetate were deleterious at pH 6·5 with 8 per cent. glucose. Acetate at 1 per cent and at 2 per cent. was favourable to citrate formation at the 14 per cent. level of glucose, but on the other hand 3 per cent. acetate exercised an unfavourable influence on the production of citrate.

The *increase* in citrate production due to 1 per cent. of acetate at the 8 per cent. glucose level represented 0·26, 0·27, and 0·28 g. C/100 ml. medium compared to controls providing a *total* citrate of 0·28, 0·32, and 0·35 g. C/100 ml. The extra citric acid thus corresponded to an 86 per cent. increase over that of the controls and represented 62 per cent. of the added acetate carbon, all of which was consumed. The increase in citric acid due to 1 per cent. of acetate at the 14 per cent. glucose level was 0·15, 0·42, and 0·21 g. C/100 ml. medium and contributed a rise of 66–210 per cent. on the citrate from cultures containing glucose alone. As above, the variation appeared to be a function of the different initial pH values. As the extra C supplied by 1 per cent. acetate amounted only to 0·4 g. C/100 ml., this indicates that (1) most of the acetate was efficiently converted to citrate, or (2) acetate was enhancing the synthesis

Table II

Effect of different levels of acetate on glucose metabolism

Results are given in g. Carbon/100 ml. solution. Treatments are indicated thus: C = control, F = pH 5·5, G = pH 6·0, H = pH 6·5. Numerals preceding letters indicate initial concentrations of acetic acid in g./100 ml.

	Initial (0 days)			Used after 10 days				Synthesized	
Treatment	Glucose carbon	Acetate carbon	Total carbon	Glucose carbon	Glucose used as % of initial glucose	Acetate carbon	Total carbon used	Citrate carbon	Oxalate carbon
8% glucose									
CF				2·58	79		2·58	0·28	0·012
CG		0	3·24	2·95	91	0	2·95	0·32	0·010
CH				2·42	75		2·42	0·35	0·013
1F		0·42	3·66	3·15	97	0·42	3·57	0·54	0·066
1G	3·24	0·44	3·68	3·22	99	0·44	3·66	0·59	0·115
1H		0·46	3·70	3·22	99	0·46	3·68	0·63	0·119
2G		0·84	4·08	3·22	99	0·84	4·06	0·34	0·202
2H		0·86	4·10	3·21	99	0·86	4·07	0·10	0·188
3H		1·23	4·47	3·21	99	1·23	4·44	0·02	0·294
14% glucose									
CF			5·83	2·75	47		2·75	0·22	
CG		0	5·83	2·71	47	0	2·71	0·20	0
CH			5·83	2·76	47		2·76	0·32	
1F		0·39	6·22	5·54	95	0·39	5·93	0·37	0·03
1G	5·83	0·40	6·23	5·70	98	0·40	6·10	0·62	0·03
1H		0·40	6·23	5·65	97	0·40	6·05	0·53	0·01
2G		0·83	6·66	3·81	65	0·60	4·41	0·34	0·02
2H		0·78	6·61	5·61	96	0·78	6·39	0·56	0·08
3H		1·23	7·06	2·31	40	0·67	2·98	0·21	0·02
0% glucose									
1G		0·42	0·42	0	—	0·35	—	—	0·19
1H		0·41	0·41	0	—	0·31	—	—	0·17
2G	0	0·75	0·75	0	—	0·72	—	—	0·29
2H		0·76	0·76	0	—	0·72	—	—	0·25
3H		1·07	1·07	0	—	1·04	—	0·01	0·40

of citrate from glucose. The same effect was also manifest with 2 per cent. acetate at pH 6·5 in the presence of 14 per cent. glucose.

Under other conditions, notably with 2 and 3 per cent. acetate at the higher pH reactions, large amounts of C (acetate and glucose) were consumed but relatively small yields of products were obtained. That other acids typical of the metabolism of *Aspergillus* species were not formed in appreciable amounts was shown chromatographically (see below).

The effect of glucose on oxalate formation from acetate was also marked. With 8 per cent. glucose very little oxalic acid was produced in the absence of acetate (Table II), whereas yields of oxalate increased progressively with higher initial concentrations of acetate in absence of glucose (Tables II and III). With 14 per cent. glucose even less oxalate was produced in the cultures containing only glucose than in the comparable treatments with 8 per cent. glucose (cf. Allsopp, 1950). The same inhibitory action of glucose on oxalate formation, however, was observed in the mixed glucose-acetate media. The results are found in Table III.

TABLE III

Effect of glucose on oxalate formation from acetate

Initial acetate %	Initial pH	Oxalate carbon formed in g./100 ml. solution after 10 days growth: Glucose 0%	Glucose 8%	Glucose 14%
0	5·5	0	0·01	0
0	6·0	0	0·01	0
0	6·5	0	0·01	0
1	5·5	No growth	0·07 (0·42)	0·03 (0·39)
1	6·0	0·19 (0·35)	0·12 (0·44)	0·03 (0·40)
1	6·5	0·17 (0·31)	0·12 (0·46)	0·01 (0·40)
2	6·0	0·29 (0·72)	0·20 (0·84)	0·02 (0·60)
2	6·5	0·25 (0·72)	0·19 (0·86)	0·08 (0·78)
3	6·5	0·40 (1·04)	0·29 (1·23)	0·02 (0·67)

Bracketed figs. indicate acetate carbon used in g./100 ml.

The effect of pH on the utilization of glucose was not apparent at the 8 per cent. level (Table II) where the efficiency was high throughout. With 14 per cent. sugar and 0 or 1 per cent. of acetate there was also no effect, but with 2 per cent. acetate the lower pH was less favourable.

The acetate utilization, which was studied at both levels of sugar, was complete in all cultures with the lower glucose concentration, whereas with 14 per cent. glucose 2 per cent. of acetate at pH 6·0 was consumed less rapidly. Indeed, under the latter conditions, both acetate and glucose utilization were well below that associated with pH 6·5. Actually the conditions for metabolism at the 14 per cent. glucose concentration with 1 per cent. acetate and also at pH 6·5 and 2 per cent. acetate were so favourable that practically all (97 per cent. +) of the available acetate had been consumed by the sixth day of growth; this despite the fact that a far larger energy supply as glucose was also present. This should be contrasted with the rate of acetate metabolism shown in Table I with much less available glucose present.

The pH at the commencement of growth appeared to exert some effect on citrate formation. This was noticeable at the 8 per cent. glucose level in cultures with glucose as the only carbon source and also with glucose cultures containing 1 per cent. acetate; under such conditions the citrate yield increased with higher initial pH. The same result was achieved at the higher glucose concentration in the absence of acetate. With 1 per cent. acetate the tendency for raised citrate levels with a higher pH was not so evident—possibly because of slightly reduced sugar utilization at pH 6·5.

Oxalate production did not appear to be related to the initial pH value.

The medium from the 14 per cent. glucose cultures was analysed for other acids by paper chromatography after the technique of Lugg and Overell (1947). In the series without acetate, citric and gluconic acids were recognized at 6, 7, and 10 days with succinate also appearing at the last day. When 1 per cent. acetate was present, citrate, succinate, and fumarate were detected on the tenth day. Succinate was also identified in cultures which initially

contained 2 per cent. acetate. An ethereal extract of the culture with an original concentration of 3 per cent. acetate was made on the tenth day and yielded malate, succinate, fumarate, glycollate, and an unidentified acid with an R_F between succinate and fumarate.

DISCUSSION

Bernhauer and Siebenauger (1931) found that when acetate or glycollate together with malate was supplied to *A. niger* both the C_2 compounds were converted to citrate but the yields were higher from acetate. Employing a similar organism, Ciusa and Brull (1939) have shown that the addition of malic or of glycollic acid, or of both acids, to sugar solutions at pH 3·5, increased greatly the formation of citric acid. On the other hand, Simola and Alapeuso (1941) observed that a mixture of pyruvic and malic acids as substrate for this mould was more effective than either acid alone. Acetate and malate had very little effect on the yield of citrate.

In the present investigation it was found that under suitable conditions acetate increased the rate of oxidation of glucose. As the acetate was also totally consumed the increases in the yields of citric acid must have been due to its formation from acetate and/or from the extra sugar utilized. Several mechanisms would appear to be available for citrate synthesis from acetate (Foster and Carson, 1950; Lewis and Weinhouse, 1951), but the present experiments do not afford any evidence which would enable us to conclude which one of these was the more likely. If citrate formation takes place simultaneously from the extra glucose utilized and the acetate consumed we could expect either substrate to provide a C_4 or C_2 unit for further condensation leading to citric acid. It is certainly evident that the mode of utilization of a portion of the used acetate in mixed glucose-acetate cultures is closely linked to the processes which give rise to citric acid formation from sugar. This was well shown by the effect of glucose on acetate metabolism (Table III) in which the oxidation of acetate to oxalate was progressively reduced with increasing levels of sugar.

Under another set of experimental conditions only small accumulations of citrate or oxalate or both acids were obtained despite the utilization of large amounts of glucose and acetate. The absence of appreciable concentrations of other products such as the familiar C_4 acids of mould metabolism has already been remarked upon. Malic acid which was readily separated from cultures growing solely on acetate was never obtained from glucose-acetate media except after exhaustive extraction of the solution. The above results suggest that an efficient oxidative system, possibly the tricarboxylic acid cycle, was present in these cultures whereby almost complete combustion of glucose and acetate occurred. This would be similar to the conditions prevailing in the experiments of Lewis *et al.* (1951). On the other hand, it appears that the increased rate and amount of citric acid synthesis in some of our cultures could be accounted for by the conversion of glucose to pyruvate which, by a carboxylation reaction, may yield oxalacetate. The latter then presumably

condenses with a C_2 unit, acetate (already present in our experiments), to form citric acid. In view of the large accumulations of citrate by strains of *Aspergillus* used in industry it remains for further investigation to show how closely the tricarboxylic acid cycle is related to the metabolism of this organism.

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The Fine Structure of Bamboo Fibres

II. REFRACTIVE INDICES AND WALL DENSITY

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SUMMARY

In the fibres of *Dendrocalamus strictus* and *D. longispathus* it has been shown that the refractive indices of the outer wall lamella, parallel and perpendicular to cell length, are correlated not only with cell length but also with the density of the whole wall. The indications nevertheless are still that the variation in refractive indices with cell length are due mainly to changes in the orientation of the cellulose microfibrils. There is, however, a subsidiary effect of factors associated with density. These factors are briefly discussed.

INTRODUCTION

In the first paper of this series (Preston and Singh, 1950) it has been shown that the precise details of wall organization in bamboo fibres vary both within a single fibre and from fibre to fibre. Thus each fibre is lamellated; in the outermost lamella the chains of cellulose run in a fairly flat spiral making an angle of some 35° to the cell length, and within the fibre there are other lamellae in which the spiral is rather steeper than this. All these lamellae are rather thin and appear bright in transverse section between crossed nicols. Between them, broader lamellae occur in which the spirals are much steeper, making angles of 5–6° (10° in *Melocanna bambusoide*). The same type of structure, with the exception of the inner bright layers, has also been shown to occur in conifer tracheides and in wood fibres (Wardrop and Preston, 1947, 1951) and in sisal fibres (Preston and Middlebrook, 1949) by equally unambiguous physical determinations on untreated material and, in fact, there is a good deal of evidence in the literature, not always, however, irreproachable from the physical point of view, to show that the same type of structure is common to all fibrous cells of higher plants. The structure of both types of lamella is related to fibre length as in the tracheide (Preston, 1948; Preston and Wardrop, 1949) in the sense that the spirals in all layers are steeper the longer the fibre.

The condition in the outer lamellae is particularly interesting in view of the work of Hermans (1946). The structure has been worked out by determining the refractive indices of this lamella both in transverse and in longitudinal section when, by assuming the units in the wall to be uniaxial crystals, both the average intrinsic birefringence and the average tilt of the optic axis to cell length could be determined. By making the further assumption that this intrinsic birefringence is invariate from fibre to fibre, a value for the tilt of the cellulose micelles to the cell length could be determined for each fibre length. The observations showed that the birefringence in longitudinal view

increases as fibre length increases, and this is a feature which might be expected to represent the common condition in natural fibres. Now Hermans (1946) has recorded data on the birefringence of regenerated cellulose fibres from which he deduces certain relationships between longitudinal birefringence and density, and in the series of fibres chosen he also includes ramie and cotton. Although this relationship is by no means strict, in view of the fact that longitudinal birefringence clearly varies with cell length it becomes pertinent to inquire how far a relationship of birefringence with density could be upheld in natural fibres, i.e. how far the change in longitudinal birefringence can be associated with tilt of the cellulose micelles (and hence to cell length) and how far with the density of the cellulose. It is, of course, to be stressed that the birefringence refers to the outermost lamella only, while the density must be determined for the whole wall, and it was expected therefore that correlation of birefringence with density would be very small if not actually zero. This paper presents relevant data for bamboo which show, in fact, that this expectation is not realized. There is indeed close correlation between birefringence, cell length, and density which has a bearing of some importance in the problem of the finer details of wall architecture.

MATERIALS AND METHODS

Two species, *Dendrocalamus strictus* and *D. longispathus*, were chosen as showing wide variation in fibre length. Delignified, macerated fibres were prepared as described in the first paper of this series (Preston and Singh, 1950), and for birefringence determinations fibres were selected for length by hand and the longitudinal refractive indices determined by the method already discussed (Preston and Singh, 1950). This method of fibre separation proved too laborious for density determinations and therefore fractionation was for this purpose achieved through a series of sieves.[1] Birefringence determinations were made as described in the first paper.

On account of the relatively small amount of material available, density determinations were made by the flotation method, using a modification of the method described by Hermans (1946). It is obviously essential that these determinations should be made on material as nearly as possible in the condition obtaining for the refractive indices measurements. To this end fibres were used dry over phosphorus pentoxide, and densities were determined by flotation in non-penetrating organic liquids. The method used was as follows.

A pellet of fibres was placed in a bulb at one end of a glass tube carrying at the other end a similar bulb containing phosphorus pentoxide. The system was evacuated and sealed and the fibres were maintained at a temperature of $105° \pm 0{\cdot}5°$ C. for 24 hours. The bulb containing the fibres was then sealed off and placed in a tube (A, Fig. 1) under carbon tetrachloride and the whole apparatus was evacuated, any traces of water vapour being absorbed in the phosphorus pentoxide bulb B. The fibres were then released by breaking

[1] We are indebted to Dr. d'A. Clark, Washington, D.C., U.S.A., for this fractionation.

the bulb by means of the steel cylinder D, actuated by means of a flexible wire passing out to the atmosphere through a mercury column E. Nitrobenzene was carefully titrated into the carbon tetrachloride by means of the taps F and G, the liquids being mixed by agitation of the steel cylinder, until the fibres remained in suspension without detectable mass movement for several hours. The density of the liquid mixture was then determined by the usual specific-gravity bottle method.

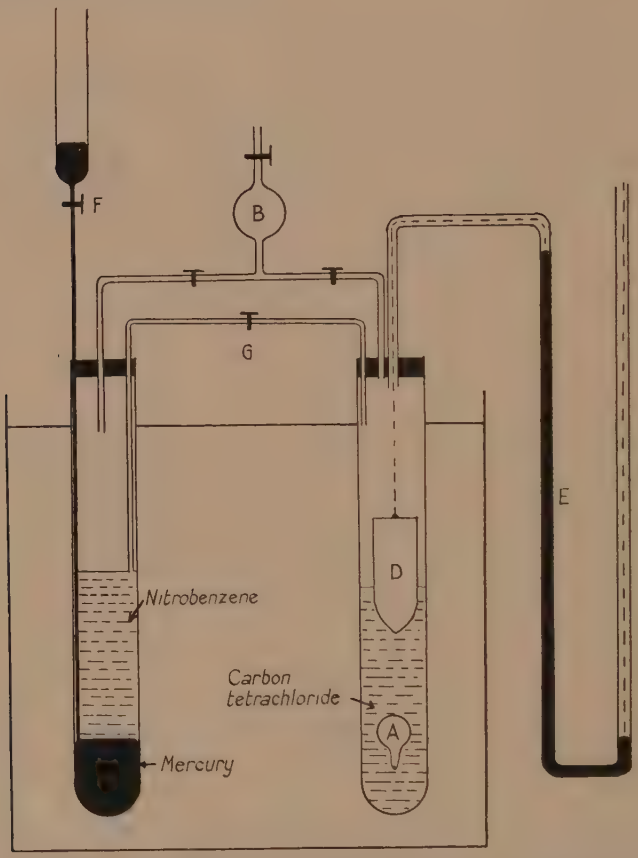

FIG. 1. Outline diagram of the apparatus used for density determinations. The whole system is immersed in a water bath at 20° ±0·1° C. For explanation see text.

RESULTS

The results are presented in Table I and in Figs. 2 to 5, where it will be abundantly clear that the refractive indices, the density (d), and the length (l) are closely interrelated. There is no clear ground for separating the two species in this respect except perhaps for the length/density relationship. Thus $n_\gamma^{\parallel}$, the refractive index for light vibrating parallel to cell length, is related to l (Fig. 2) in approximately the form

$$n_\gamma^{\parallel} - 1 = 0{\cdot}5715 + 0{\cdot}004l.$$

This may be compared with the relation

$$n_{\gamma}^{\parallel} - 1 = 0{\cdot}565_9 + 0{\cdot}003_6 l$$

calculable from the data presented in the previous paper for the same material using hand-selected fibres with closer control, therefore, of fibre length within

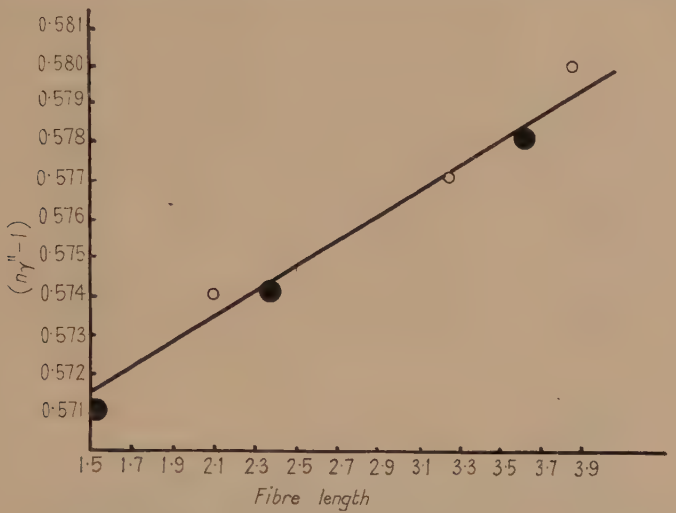

FIG. 2. —○— *Dendrocalamus longispathus*; —●— *D. strictus*. For explanation see text.

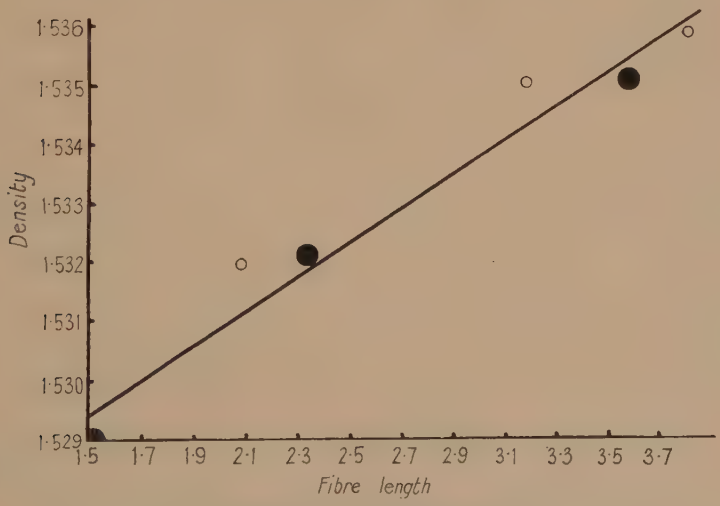

FIG. 3. —○— *Dendrocalamus longispathus*; —●— *D. strictus*.

each sample. It can be interpreted, as was done in the first paper, following the demonstration of a spiral organization in the outer layers of these fibres, in terms of a steepening of the spiral as the length increases. Assuming the intrinsic birefringence to be invariate, this gives a relation between spiral

angle and length in a form closely similar to that known to obtain in other cells (Preston, 1948; Preston and Wardrop, 1949). Since, however, fibre

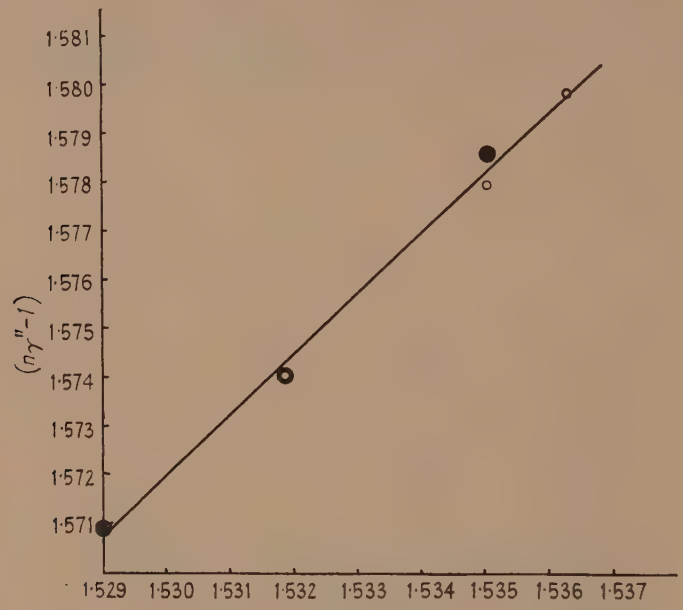

FIG. 4. —○— *Dendrocalamus longispathus*; —●— *D. strictus*.

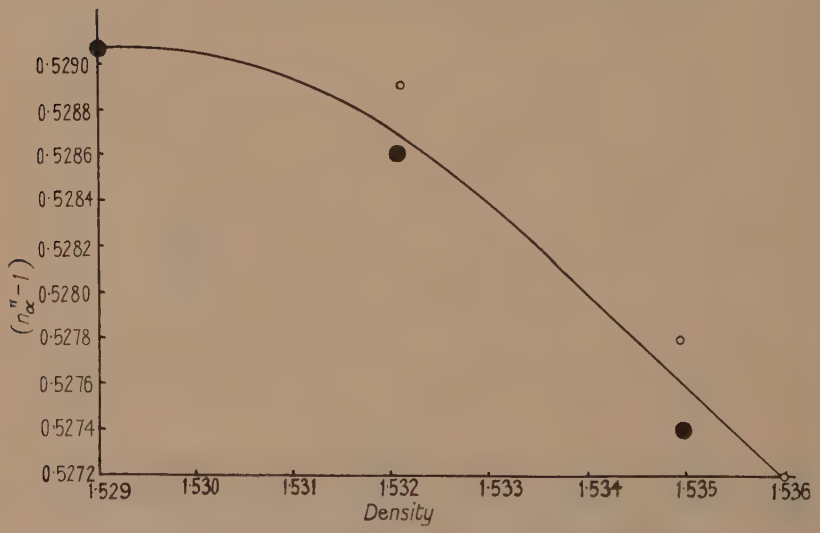

FIG. 5. —○— *Dendrocalamus longispathus*; —●— *D. strictus*.

length and wall density are linearly related (Fig. 3), it necessarily follows that n_{γ} and density are also related (Fig. 4) and it remains to be seen if the n_{γ}/l relationship could be interpreted in some way not involving a change in spiral angle.

DISCUSSION

We note first that $n_\alpha^{\parallel}$, the minor refractive index of the fibre edge seen in longitudinal view, and d are related in a form which could not be explained merely in terms of a change in spiral angle (Fig. 5), so that the change in $n_\alpha^{\parallel}$ can be associated with change in d rather than in l, in spite of the fact that $n_\alpha^{\perp}$ refers to the outer layer only while d refers to the whole wall. Consequently some part at least of the variation of $n_\gamma^{\perp}$ will be associated purely with density change.

There seem at the moment only two ways in which change in wall organization might effect the necessary changes in $n_\gamma^{\parallel}$, $n_\alpha^{\parallel}$, and d: changes in the crystalline/non-crystalline ratio with length and changes in the angular dispersion with length. Taking the former possibility first, there is some slight evidence that the crystalline/non-crystalline ratio does increase with cell length (Preston, Hermans, and Weidinger, 1950) so that, although again this refers to the whole wall in bulk, the possibility of a similar change in the outer layer itself has to be taken seriously. The merits of such a possibility in explaining the present results can fortunately be tested, if somewhat crudely.

If n_γ, n_α be the two refractive indices of the fibre edge in longitudinal section ($n_\gamma > n_\alpha$), n_γ, n_α the intrinsic refractive indices of the cellulose (i.e. parallel and perpendicular to the common run of the cellulose chains), and θ the angle which the chains make with cell length, then it can be shown that

$$n_\gamma = \frac{n_\gamma^{\parallel} n_\alpha^{\parallel} \cos\theta}{\sqrt{\{(n_\alpha^{\parallel})^2 - (n_\gamma^{\parallel})^2 \sin^2\theta\}}}.$$

If, further, f is the fraction of the wall which is crystalline in the optical sense then, provided the mixture formula holds,

$$n_\gamma - n_\alpha = f(n_{\gamma c} - n_{\alpha c}),$$

where the subscript c refers to the crystalline component only. Since $n_\alpha = n_\alpha^{\parallel}$, it follows that

$$\frac{n_\gamma^{\parallel} n_\alpha^{\parallel} \cos\theta}{\sqrt{\{(n_\alpha)^2 - (n_\gamma)^2 \sin^2\theta\}}} - n_\alpha^{\parallel} = f(n_{\gamma c} - n_{\alpha c}).$$

Now if we take θ invariate at 34°, then, for the shortest and longest fibre in Table I this relation gives

shortest fibre $\quad f(n_\gamma - n_\alpha) = 0{\cdot}0625,$

longest fibre $\quad f(n_\gamma - n_\alpha) = 0{\cdot}0790.$

Since the recorded values for $(n_\gamma - n_\alpha)$, even making allowance for the factor f, are never higher than 0·07, it follows that θ is not in fact invariate but must be smaller than 34° with the longer fibres. The effects here, in other words, cannot solely be expressible in terms of changes in percentage crystallinity.

The possible effects of changes in angular dispersion with length cannot be so easily assessed, for until we know more about the packing of the cellulose microfibrils it is impossible to derive a formula which expresses satisfactorily birefringence in terms of angular dispersion. Nevertheless we can make an approximation by assuming, in any particular case, that all the micelles lie at an average angle α to their mean orientation. The value of α can then be deduced from the value of $n_\alpha^\perp$ and the corresponding $n_\gamma^\parallel$ calculated. This can then be compared with the observed value.

TABLE I

Refractive indices and density in bamboo fibres

Fibre species	Length (mm.)	$n_\gamma^\parallel$	$n_\alpha^\perp$	Density
Dendrocalamus longispathus	2·10±0·01	1·574$_2$	1·528$_9$	1·532
	3·15±0·10	1·577$_9$	1·527$_8$	1·535
	3·78±0·13	1·580$_1$	1·527$_2$	1·536
D. strictus	1·51±0·07	1·571$_0$	1·529$_5$	1·529
	2·35±0·09	1·574$_0$	1·528$_6$	1·532
	3·57±0·09	1·578$_6$	1·527$_4$	1·535

Thus, let us take the smallest value observed for $n_\alpha^\perp$ (1·526) for cells 5 mm. long as representing undispersed cellulose (Preston and Singh, 1950). For these cells $n_\gamma^\parallel = 1{\cdot}584$, and it can be calculated, using equation (1) above, that $n_\gamma = 1{\cdot}614$. This is already markedly higher than any value recorded for cellulose, but it may be accepted for the moment. Turning now to cells 1 mm. long we have $n_\alpha^\perp = 1{\cdot}530$. The angle of dispersion as defined above to give this value of $n_\alpha^\perp$ would be given by

$$\frac{(1{\cdot}530)^2 \sin^2\alpha}{(1{\cdot}614)^2} + \frac{(1{\cdot}530)^2 \cos^2\alpha}{(1{\cdot}526)^2} = 1,$$

i.e. $\alpha = 13{\cdot}6°$.

It is to be noted that in this calculation the dispersion is supposed to occur only about a line lying in the wall surface and perpendicular to the 'mean' direction. The corresponding angle for uniform dispersion would be slightly greater than this. At this angle, n_γ would be reduced to a value which can be calculated as $n_\gamma = 1{\cdot}608$. Remembering that this is tilted at an angle of 34° to the length of the cell we have finally $n_\gamma^\perp = 1{\cdot}581$. This is considerably higher than the value recorded for these cells (1·569) and, despite the crudity of the conceptions underlying the calculations, makes it very doubtful whether changes in angular dispersion alone could harmonize the changes in $n_\alpha^\perp$ with those in $n_\gamma^\parallel$ as the cells increase in length. Further, since assumed changes in angular dispersion and in crystallinity each individually lead to values for n_γ at the upper limit of known values for cellulose and even exceeding them, there seems no point in attempting further explanation along these lines in terms of any combination of both. It seems clearly demonstrated that the changes of $n_\gamma^\parallel$ with length are due largely to changes in net orientation.

Certainly the lower dispersion or the greater crystallinity associated with higher density in longer fibres would imply that the steepening of the cellulose spiral with length increase is slightly less rapid than was at first thought, though the effect of these variables upon the calculated angles is of secondary importance only.

There would seem to remain therefore a correlation of spiral angle not only with length but also with wall density, and this is worthy of some further thought. The parallel behaviour of the spiral in the outer layer and the density of the whole wall may be taken to reflect the concomitant behaviour of the spiral angle in all wall layers. Now hitherto it has been tacitly assumed that, since the fibre reached its final length, or thereabouts, before any secondary wall thickening had occurred, then the spiral angle was governed by some cytoplasmic mechanism either responsive directly to dimension changes or associated with some other factor under dimensional control. The question now naturally arises as to the position of wall density in such a scheme. Is density an intermediate factor or does the density vary among fibres of different length only because the spiral angle varies, i.e. is the scheme

length—metabolic factor (?)—density—angle

or length—geometric factor—angle—density?

The former scheme would imply that the denser packing of the microfibrils on a cylindrical surface involves an orientation more nearly parallel to cell length, for which there seems at the moment no evidence and little reason. The latter would imply that microfibrils laid down at a low spiral angle on a cylindrical surface are incapable of the close packing which obtains with steeper angles. Although at the moment we are tempted to accept the latter alternative, it is clear that questions of this kind cannot receive an answer until more data are available.

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The Analysis of the Alcohol-insoluble Nitrogen of Plants by Quantitative Procedures based on Paper Chromatography

I. THE ANALYSIS OF CERTAIN PURE PROTEINS

JOHN F. THOMPSON

II. THE COMPOSITION OF THE ALCOHOL-SOLUBLE AND INSOLUBLE FRACTIONS OF THE POTATO TUBER

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SUMMARY

Part I. The amino-acid composition of egg albumin, zein, edestin, cucumber (*Cucumis*) seed globulin, and squash (*Cucurbita*) seed globulin has been determined by a quantitative paper chromatographic method. The chromatographic analyses account completely for the amino-acid nitrogen of the protein hydrolysate as determined by the Kjeldahl method. More complete analyses than hitherto of cucumber and squash seed globulin have been obtained. These analyses can now be regarded as complete since histidine and tryptophane, which have not been determined chromatographically, have previously been reported. The two cucurbit seed globulins analysed closely resemble edestin, and evident differences between these three on the one hand and zein on the other are referred to in the text. It is evident that the paper chromatographic method is applicable to the quantitative amino-acid analysis of the protein-N fraction of the plants as well as to the analysis of pure proteins.

Part II. The analysis of the alcohol-soluble and insoluble fractions (hydrolysate) of potato tubers of the variety 'Sebago' has been carried out. Attention is drawn to differences which exist between the samples and varieties which have been investigated, namely 'King Edward', 'Sebago', and 'Katahdin'. These differences relate particularly to the relative proportion of soluble to insoluble nitrogen, to the amount of amides present in the soluble fraction, and to the ratio of asparagine to glutamine.

There is no correlation between the relative proportions of amino-acids as they exist free in the tuber and as they occur in the alcohol-insoluble (protein) fraction. Certain compounds (e.g. γ-aminobutyric acid) are present free in the tissue, but they do not occur in the protein. Also the amides far exceed their possible occurrence combined in the protein. Proline especially is much more abundant in the combined than in the free state, and the ratios between the various free amino-acids are quite different from those which apply to the protein. The latter data suggest that the soluble compounds which occur free are not directly combined as such to form protein.

The utility of the quantitative procedure based on partition chromatography on paper in the analysis of the alcohol-insoluble (protein) fraction of plants is, therefore, established.

INTRODUCTION

PARTITION chromatography on paper has greatly enlarged our outlook on the composition of the alcohol-soluble nitrogen fraction of plants in which the amino-acids and amides predominate (Steward and Thompson, 1950). It has done this in two principal ways. First, it has made it possible to show that the range of identifiable substances which can occur in this fraction at any one time is far greater than might otherwise have been supposed. This is particularly true of certain storage organs in which the soluble-N may predominate over protein-N. Secondly, paper chromatography has been the means by which substances, not commonly regarded as components of the alcohol-soluble nitrogen of plants, have been recognized (e.g. β-alanine, γ-aminobutyric acid).

Indeed, it is now evident that the full range of the natural ninhydrin reacting compounds which exist in plants, and the changes they undergo, is only just becoming apparent. The quantitative procedure which the authors have recently described (Thompson, Zacharius, and Steward, 1951; Thompson and Steward, 1951) now permits the accurate determination of any soluble constituent which can be chromatographed, identified, and which reacts with ninhydrin on paper.

However, the nitrogen metabolism of plants cannot be comprehended fully unless the alcohol-insoluble fraction, predominantly protein, also yields to similar methods which will permit one to record the changes in the relative proportions of its constituent amino-acids. In this way it would become possible to ascertain whether the composition of the protein synthesized under a variety of nutritional and environmental conditions, or even at different stages of development, is always the same. It may even be anticipated that differences in the protein-N fraction, correlated with nutritional, environmental, or developmental conditions, may be revealed by their amino-acid analysis. Ideally the alcohol-insoluble fraction should be resolved into its constituent pure proteins, each of which could be separately analysed. As a first step, however, it will be instructive to determine the composition of the protein fraction as a whole and to compare it with the alcohol-soluble fraction. This communication will show that such analyses may be made on relatively small amounts of material (approximately 50 μg. of alcohol-insoluble N): this being so, a number of problems may now be attacked.

It should now be possible to trace the changes which occur in the composition of the protein fraction as development proceeds behind the apex of shoot and root. The influence of neutral salts on protein synthesis in thin disks of storage tissue is well known (Steward and Preston, 1940, 1941 *a*, *b*): the methods to be described will enable one to determine the effect of the salts on the *composition* as well as the *amount* of new protein synthesized. A rapid micro method of determining the amino-acid composition of protein will obviously find numerous applications in plant physiology and in experimental morphology. To mention but a few: do the proteins in the

meristematic cells of apical growing-points in any way reflect the different potentiality for growth of shoot and root? or of the vegetative and floral shoot? or of the etiolated and normal shoot? When electrophoretically homogeneous proteins can be isolated from these contrasted growing regions (as some workers are now doing from leaves; Wildman and Bonner, 1947) their amino-acid composition will be illuminating. Even lacking this refinement, however, much may be learned if there is a method of determining the amino-acid composition of relatively small composite samples of protein. This paper aims to prove that this can be done with sufficient accuracy to make feasible investigations of the kind which are mentioned above.

Knowledge of the amino-acid composition of plant proteins has until recently been based largely on the reserve or storage proteins which have been isolated and purified and which are available in large quantity. Even the number of these for which accurate or complete analyses are available is small, e.g. edestin, zein, gliadin (Tristram, 1949). The emphasis on seed proteins is obvious. Analyses of the amino-acid composition of reserve or storage proteins can have only a limited interest for the student of metabolism, whose attention turns rather to the more physiologically active proteins of growing regions or of actively metabolizing cells generally.

The experimental work of this paper will be reported in two parts. The first will show that accurate analyses of the amino-acid composition of pure proteins may be obtained by methods based on paper chromatography. The second will compare the amino-acid composition of the alcohol-soluble and insoluble fraction of a familiar material, namely, the potato tuber.

I. THE ANALYSIS OF CERTAIN PROTEINS BY QUANTITATIVE PAPER CHROMATOGRAPHY

Since T. B. Osborne isolated pure plant proteins around the turn of the century, much effort has been expended on the analysis of the amino-acids obtained from protein hydrolysis.

The amino-acids which have functional groups, other than amino and carboxyl groups, can be measured in special ways by specific chemical methods, e.g. the Pauly reagent for histidine and others (Block and Bolling, 1951). The amino-acids with no extra functional groups can only be measured by chemical methods after their somewhat difficult separation and isolation or by the use of isotope dilution methods.

Biological methods for amino-acid analysis are now in general use. In principle the bio-assay method is one in which the growth of a selected micro-organism is governed by the supply of a particular amino-acid, all other known essentials for growth being in excess. The amount of growth thus becomes a measure of the amino-acid to be determined. To a lesser degree, specific enzymatic methods have also been employed.

Within the past decade the means of separating the amino-acids of a mixture by means of partition chromatography on paper have been developed

(Consden, Gordon, and Martin, 1944). Stein and Moore (1948) have also worked out a quantitative procedure for analysis of amino-acids by chromatography on a starch column. While the latter technique has yielded accurate results, it is somewhat cumbersome for many purposes. In order to investigate the nitrogen metabolism of plants, a quantitative paper chromatographic method has been developed (Thompson, Zacharius, and Steward, 1951, and Thompson and Steward, 1951). The particular feature of this work was the close attention given to the variables involved in both the ninhydrin reaction on paper and in the chromatography and to their control in the quantitative procedure which has been devised and tested.

To demonstrate the applicability of the quantitative paper chromatographic method to the analysis of the protein-N fraction of plants, analyses have been made of certain proteins, the amino-acid analyses of which have often been made by other means. An analysis of casein by this method has already been reported (Thompson and Steward, 1951). Analysis of five other proteins are here presented and compared with published data. It will become apparent that the paper chromatographic method has produced a nearly complete analysis, and in certain respects even a more accurate one than has been obtained hitherto.

The proteins which were analysed were egg albumin, zein, edestin, cucumber (*Cucumis sativus* var. 'Davis Perfect') seed globulin, and squash (*Cucurbita maxima* var. 'Hubbard') seed globulin. Egg albumin, zein, and edestin, being easily isolated, are available in pure form and have been analysed frequently. The two seed globulins were prepared in a state of purity by Vickery, Smith, Hubbell, and Nolan (1941) in order to obtain an easily available alternative globulin to edestin. The purified seed globulins were supplied to us by Dr. H. B. Vickery of the Connecticut Agricultural Experiment Station. Partial amino-acid analyses of these proteins have been made by Dr. E. L. Smith and his co-workers (1946, 1947, 1948). The cucumber seed globulin contained 18·5 per cent. nitrogen; the squash seed globulin had a nitrogen content of 18·55 per cent. of the dry protein.

Each protein was hydrolysed in 6N.HCl (50 ml. per g. of protein) for 16 hours at 120° C. The HCl was removed by distillation *in vacuo*. The residue was dissolved in water and neutralized with NaOH to pH 6·0. This was filtered and the volume adjusted so that there were approximately 2 mg. of nitrogen per ml. This solution was analysed for total nitrogen by the Kjeldahl procedure and for ammonia (Pucher *et al.*, 1935). The protein hydrolysates were analysed by the quantitative paper chromatographic method already mentioned.

Before presenting the data as obtained, certain general comments may be made.

The sulphur-containing amino-acids (cystine and methionine) present somewhat special problems. It will also be noted that the values obtained by the paper chromatographic method deviate somewhat from those reported by other methods. The sulphur acids are measured as their oxidation products

(cysteic acid and methionine sulphone respectively). This oxidation is still difficult to perform quantitatively. Also the small amount of these acids present (which is well below 5 per cent. of the total in the protein) renders their determination more difficult.

The determination of proline suffers because the blank represents a rather large part of the ninhydrin colour produced by the proline so that the blank correction is relatively large.

All the serine and threonine values have been corrected for losses due to hydrolysis according to the work of Rees (1936).

The paper chromatographic procedure did not lend itself to the determination of histidine for two reasons: first, the reaction between this acid and ninhydrin is not very sensitive, and, second, it was present only in very small amounts.

Tryptophane was, of course, not determinable in the acid hydrolysate of the protein and alkaline hydrolysis was not resorted to.

It was not regarded as important enough in this first study to employ the particular solvent combinations which will separate leucine and *iso*leucine on paper: the term 'leucines' is used to designate the combined leucine and *iso*leucine in the samples analysed (see Tables I to IV).

Analysis of egg albumin

There are several points of interest in the analysis of egg albumin obtained by the paper chromatographic method (Table I).

There is some discrepancy between different published analyses (Table I, columns 3, 4, and 5) and the more recent values (column 5 and some of those in columns 3*b* and 4) should be the more accurate. The more recent analyses agree with several of the chromatographic results, viz. aspartic acid, glycine, lysine, leucines, and phenylalanine, more closely than with previously published analyses (column 3*a*). For these amino-acids the chromatographic values are undoubtedly more accurate than those obtained by older methods. The figures for arginine and tyrosine are consistent throughout. Even recent analyses (columns 3*b*, 4, and 5), however, differ in the values for glutamic acid, alanine, proline, and valine. In the case of alanine and proline, the chromatographic results agree well with those of Lewis *et al.* (1950).

Some of the values preferred by Block and Bolling (column 3*b*) may be somewhat arbitrary. In the case of proline, Block and Bolling prefer one value obtained by a microbiological method (1951, p. 372) which gave a value much higher than those obtained by other methods (column 4) and by the microbiological procedure of Lewis *et al.* (1950). The valine figure of 6·2 per cent. was evidently arrived at by Block and Bolling (1951, p. 307) as the mean of four values (obtained by microbiological methods) which range from 3·9 to 7·3 per cent., whereas the mean of the three most concordant values would give an average of 6·9 per cent., which is in close agreement with the chromatographic value of 7·0 per cent. and the figure chosen by Tristram

(1949). The valine value of 8·8 per cent. reported by Lewis *et al.* (1950) may be too high, for it does not agree with other determinations by similar methods (Block and Bolling, 1951).

TABLE I

The amino-acid composition of egg albumin

Grammes of amino-acid from hydrolysis of 100 g. of dry protein

Column 1	Column 2	Column 3		Column 4	Column 5
	Analysis by paper chromatographic method, Thompson and Steward (1951)	Analysis compiled by Block and Bolling		Analysis compiled by Tristram (1949)	Analysis performed by Lewis *et al.* (1950)
Amino-acid		3*a* (1947)*	3*b* (1951)†		
Cystine	1·6	1·9–2·9‡	2·3	1·9	—
Aspartic acid	9·3	7·9	8·7	9·3	9·2
Glutamic acid	15·0	15·7	15·4	16·5	15·7
Serine	5·7	7·3	8·2	8·1	8·5
Glycine	3·7	1·8	3·5	3·0	3·2
Threonine	3·4	2·9–3·9	4·0	4·0	4·0
Alanine	6·9	7·1	7·1	6·7	5·7
Histidine	—	2·3	2·3	2·3	2·4
Lysine	6·7	7·4	6·3	6·3	6·4
Arginine	5·6	5·5	5·9	5·7	5·9
Methionine	3·0	4·8	5·3	5·2	5·4
Proline	3·2	3·8–4·8	7·8	3·6	3·8
Valine	7·0	6·6	6·2	7·0	8·8
Leucine	—	9·1	9·1	9·2	9·9
Isoleucine	—	—	7·2	7·0	7·1
Leucines	14·5	9·1	16·3	16·2	17·0
Tryptophane	—	1·3	1·4	1·2	—
Phenylalanine	7·8	5·8	7·2	7·6	7·5
Tyrosine	4·2	4·0	4·0	3·7	3·8
Total	95·3	95·2–98·2	111·9	109·8	107·3

* Recalculated to 15·4% nitrogen (from data of Block and Bolling, 1947, p. 301).
† Recalculated to 15·4% nitrogen (from data of Block and Bolling, 1951, p. 487).
‡ On p. 180, loc. cit., there appears an aberrant value of 1·6.

The analytical results obtained for serine, threonine, and the leucines by the chromatographic method are lower than recent values (columns 3*b*, 4, and 5). Even if the serine value obtained by chromatography is multiplied by the larger factor 1·34 (suggested by Nicolet *et al.* (1942) for casein) the serine value is still only 6·8 per cent., which is considerably below the value of 8·5 per cent. obtained by Lewis *et al.* (1950). This indicates that the chromatographic value is still lower for unknown reasons than results obtained by other methods. Other chromatographic results (cf. Table II, Table III, and the analysis of casein (Thompson and Steward, 1951)) have also given low values for serine.

Previously published serine values have been considered to be low rather than high (cf. Gordon *et al.* (1949) and Nicolet *et al.* (1942)). However, it is

quite conceivable that the chemical method for serine (periodate oxidation) is not as specific as had hitherto been believed since formaldehyde from any other source than the action of periodate on serine would increase the apparent serine content.

Another possible explanation for seemingly low serine values by paper chromatographic methods is that some serine may be included with glycine or glutamic acid in the chromatographic separation. However, since the values for these amino-acids are not noticeably high and since clear-cut separations in the chromatograms have been consistently obtained, it is considered unlikely that the serine values are low for this reason.

There is no obvious explanation for the discrepancies (columns 2, 3*b*, and 5, Table I) in leucine values unless the samples of egg albumin were not similar.

Analysis of zein

In Table II the amino-acid analysis of zein as determined by paper chromatographic methods is compared with various published results. As in the case of egg albumin, it is assumed that, in the main, the more recent analyses those reported by Chibnall (1943), by Tristram (1949), and by Block and Bolling (1951)) are the more accurate. The chromatographic results agree best with the most recent results in several instances, namely aspartic acid, serine, threonine, arginine, valine, and proline.

In the case of alanine, the value now preferred by Block and Bolling (column 3*b*) seems to be based on the mean of a microbiological determination which gave a high value (12·3 per cent.) and Chibnall's data (10·5 per cent., see column 5). The latter agrees closely with the chromatographic results.

Differences between the analyses obtained by chromatographic methods (column 2) and other data (columns 3*b*, 4, and 5) for glutamic acid cannot be explained. It might be pointed out, however, that these discrepancies are not general since for egg albumin the glutamic acid values by chromatography and as reported in the literature were in reasonable agreement (cf. columns 2, 3, 4, and 5 of Table I). Again, in the case of the leucines, the new analysis by paper chromatography is in better agreement with the earlier ones cited by Block and Bolling (column 3*a*) than with the lower estimates of Chibnall and Tristram (columns 4 and 5) and the later ones of Block and Bolling (column 3*b*).

The presence of glycine in the hydrolysate of zein is believed to be correct although it has not been previously reported. Lower values in the determination of serine by paper chromatography were previously referred to (p. 175).

The absence of lysine in zein (columns 3 to 5 of Table II) is again confirmed. There was no trace of lysine on any chromatogram from this protein: this also agrees with the work of Zittle and Eldred (1944) and of Neuberger (1945) using lysine decarboxylase.

TABLE II

The amino-acid composition of zein

Grams of amino-acid from hydrolysis of 100 g. of dry protein

Column 1	Column 2	Column 3*a*	Column 3*b*	Column 4	Column 5
Amino-acid	Analysis by paper chromatographic method, Thompson and Steward (1951)	Analysis compiled by Block and Bolling (1947, p. 304)	Analysis compiled by Block and Bolling (1951, p. 490)	Analysis compiled by Tristram (1949)	Analysis compiled by Chibnall (1943)
Cystine	0·6	0·8	1·0	0·8	1·7
Aspartic acid	5·4	3·4	5·6	4·6	4·7
Glumatic acid	31·8	35·6	26·6	26·9	26·8
Serine	5·9	—	7·7	7·0	7·0
Glycine	1·4	0·0	0·0*	—†	—
Threonine	3·3	2·4	3·0	3·4	3·4
Alanine	10·3	9·9	11·4	10·5	10·5
Histidine	—	0·9	1·7	1·3	1·2
Lysine	0·0	0·0	0·0	0·0	0·0
Arginine	1·8	1·6	1·8	1·7	1·7
Methionine	1·5	2·0	2·3	2·4	2·4
Proline	10·6	9–12	10·4	10·5	10·5
Valine	4·0	2·4	3·0	3·5	3·5
Leucine	—	23·7	23·7	—	—
*Iso*leucine	—	4·3	7·3	—	—
Leucines	26·9	28·0	31·0	22·5	22·4
Phenylalanine	13·6	6·4	6·4	5·9	5·9
Tryptophane	—	0·1	0·1	1·0	0·2
Tyrosine	6·6	5·0	5·2	5·3	5·3
Hydroxyproline	0·0	1·0	—	—	—
Total	123·7	108·5–111·5	117·2	106·6	107·2

* Analysis performed but none of the substance was detectable.
† Analysis not performed.

The phenylalanine value in column 2 is believed to be high, due to the fact that in the presence of such a high concentration of the leucines the separation is not always complete. A small fraction of the leucines placed in the phenylalanine fraction will make a large error in the phenylalanine determination because the leucines produce, weight for weight, about five times as much colour with ninhydrin after chromatography as does phenylalanine.

The chromatographic value for tyrosine is somewhat high for unknown reasons.

Analysis of edestin

The analysis of edestin as obtained by the paper chromatographic method is compared in Table III with various published values and with the latest compilation (column 6) by Block and Bolling (1951) recalculated to an 18·6 per cent. nitrogen content. The data of Henderson and Snell (column 4) and those cited by Block and Bolling (column 6) are probably more accurate than those cited by Cohn and Edsall (column 3).

The data obtained by the paper chromatographic method (column 2) agree

with the more recent values (columns 4, 5, and 6) except for glumatic acid, arginine, and proline. There is no obvious reason for this in the case of glutamic acid and arginine. For glutamic acid, the chromatographic results have been so consistent that there is no reason to doubt them for the sample analysed. The chromatographic methionine value agrees with the value compiled by Block and Bolling but disagrees with that of Henderson and Snell. The serine and proline values by chromatography are again low, for reasons already explained (see pp. 174 and 175).

TABLE III

The amino-acid analysis of edestin

Grams of amino-acid from hydrolysis of 100 g. of dry protein

Column 1	Column 2	Column 3	Column 4	Column 5	Column 6
Amino-acid	Analysis by paper chromatography, Thompson and Steward (1951)	Analysis compiled by Cohn and Edsall (1943)	Analysis performed by Henderson and Snell (1948)	Analysis compiled by Tristram (1949)	Analysis* compiled by Block and Bolling (1951, p. 491)
Cystine	0·8	1·4	—	1·4	1·3
Aspartic acid	13·2	12·0	13·4	12·0	12·0
Glutamic acid	23·2	20·7	19·4	20·7	20·7
Serine	5·8	0·3	—	6·3	6·3
Glycine	5·1	3·8	—	—	5·1
Threonine	3·9	—	3·7	3·8	3·8
Alanine	5·4	3·6	—	4·3	5·5
Histidine	—	2·4	2·6	2·9	2·9
Lysine	2·0	2·4	2·1	2·4	2·7
Arginine	14·8	16·8	17·4	16·7	16·6
Methionine	2·9	4·1	4·6	2·4	2·3
Proline	1·0	2·4	2·1	4·2	4·6
Valine	5·5	5·6	6·6	5·7	6·3
Leucine	—	—	7·5	4·7	7·7
*Iso*leucine	—	—	6·5	7·5	4·7
Leucines	14·0	20·9	14·0	12·2	12·4
Phenylalanine	4·7	3·1	5·2	5·4	5·8
Tryptophane	—	1·5	0·9	1·5	1·5
Tyrosine	4·7	4·5	3·7	4·3	4·5
Hydroxyproline	—	2·0	—	—	—
Total	107·0	107·5	95·7	108·5	114·3

* Recalculated to 18·6 % nitrogen.

Analysis of certain seed globulins from Cucurbita

In Table IV amino-acid analyses of cucumber seed globulin and squash seed globulin are presented along with the partial analysis by Smith and Greene (1947). Wherever values are available for cucumber seed globulin by both the methods of Smith and Greene and by the paper chromatographic method (columns 2 and 3) they agree well except for phenylalanine. In addition, the analyses of cucumber seed globulin (Table IV) for six amino-acids which have not been previously determined are now presented. By this means

the sum of the amino-acids in cucumber seed globulin has been raised from 60 to 108 per cent. of the protein.

TABLE IV

Amino-acid composition of cucumber seed globulin and squash seed globulin

Grams of amino-acid from hydrolysis of 100 g. of dry protein

Column 1	Cucumber seed globulin		Squash seed globulin	
	Column 2	Column 3	Column 4	Column 5
Amino-acid	Analysis by paper chromatography, Thompson and Steward (1951)	Analysis by Smith and Greene (1947)	Analysis by paper chromatography, Thompson and Steward (1951)	Analysis by Smith and Greene (1947)
Cystine	1·2	1·1	0·8	1·1
Aspartic acid	9·3	—	6·8	—
Glumatic acid	21·4	—	24·2	—
Serine	5·1	—	5·7	—
Glycine	4·9	—	5·5	—
Threonine	3·4	3·6	3·0	2·8
Alanine	5·1	—	5·7	—
Histidine	—	2·3	—	2·2
Lysine	3·0	2·9	4·0	3·0
Arginine	15·7	15·8	15·2	16·2
Methionine	2·2	2·5	2·5	2·3
Proline	4·9	—	5·4	—
Valine	5·4	7·0	5·6	6·5
Leucine	—	9·1	—	8·0
*Iso*leucine	—	4·9*	—	5·5†
Leucines	12·8	14·0	13·3	13·5†
Phenylalanine	10·0	6·5	8·3	6·8
Tryptophane	—	1·9	—	1·7
Tyrosine	3·8	4·6	3·7	4·4
Total	108·2	60·3	109·7	58·8

* The *iso*leucine value was repeated by Smith and Greene, 1948.

† The *iso*leucine value is high because the standard DL-*iso*leucine was contaminated with DL-allo*iso*leucine.

Table IV (columns 4 and 5) presents the amino-acid analysis of squash seed globulin as determined by the paper chromatographic method and by Smith and Greene (1947). Again the agreement between the analyses is satisfactory. Values for aspartic acid, glutamic acid, serine, glycine, alanine, and proline are reported for the first time. The sum of the amino-acids in the squash seed globulin is raised thereby from 59 to 110 per cent.

The general similarity in the amino-acid composition of the cucumber seed globulin and squash seed globulin may be noted: this is consistent with their origin from closely related plants (cf. Vickery *et al.*, 1941).

One preparation of cucurbit seed globulin yielded in the hydrolysate a spot which corresponded to citrulline and which also reacted with *p*-dimethylaminobenzaldehyde (Dent, 1948). In view of the fact that other preparations did not show this, the presence of citrulline in the pure protein remains

doubtful. Citrulline has been reported (Wada, 1933) in the hydrolysate of casein: in our work on casein, however, no indications of citrulline were found.

ANALYSIS OF PURE PROTEINS: GENERAL CONSIDERATIONS

In each of the Tables I to IV the percentage composition of the given protein in terms of amino-acids is presented and in three cases the total is over 100 per cent. The percentage recovery of the amino-acid nitrogen in the solution has been calculated, and this compared with the nitrogen in the amino-acids according to the chromatographic analysis. The amino-acid nitrogen was obtained as the difference between the total nitrogen and the ammonia nitrogen in the hydrolysate. Table V shows that the recovery of amino-acid nitrogen by the chromatographic method ranged from 97 to 103 per cent. except in the case of edestin (85 per cent.). This indicates that the chromatographic analyses adequately account for the nitrogen in the protein hydrolysate. The somewhat lower recovery of amino-acid nitrogen in the case of edestin remains unexplained.

Table V

Recovery of the amino-acid nitrogen in a solution of hydrolysed protein by the chromatographic method

The nitrogen content of amino-acids as determined by chromatographic analysis was calculated. This was compared with the amino-acid nitrogen content of chromatographed solution. The amino-acid nitrogen content of the hydrolysates was determined as the difference between the total nitrogen content of the solution and the ammonia nitrogen content. The total nitrogen was determined by the Kjeldahl method and the ammonia was determined by the method of Pucher, Vickery, and Leavenworth (1935).

Protein	Percentage recovery of non-ammonia nitrogen (by Kjeldahl) as amino-acid nitrogen by chromatography
Egg albumin	103·3
Zein	98·8
Cucumber seed globulin	97·3
Squash seed globulin	100·5
Edestin	85·0

The protein hydrolysates contained ammonia-nitrogen, the amount of which was determined by the method of Pucher *et al.* (1935). Even recognizing that some of this ammonia arises from the decomposition of other acids, it is still interesting to calculate the *maximum* quantity of amide-N which could be combined in the protein and to compare this with the glutamic and aspartic acid content as found. Combined glutamine and asparagine could account in large part for the glutamic and aspartic acids liberated from the protein upon acid hydrolysis: this could also be true of the cucurbit seed globulins, in which these amides could account for up to 50 per cent. of their acids in the protein.

Certain familiar relationships reappear among the three seed globulins

(edestin and the two *Cucurbita* proteins) as well as certain evident contrasts with the prolamine (zein).

The content of arginine in the seed globulins is much higher than in zein. In the seed globulins, arginine accounts for approximately 25–30 per cent. of the total nitrogen, whereas in zein it is only about 5 per cent. Zein is relatively richer than all the seed globulins in the following: glutamic acid, alanine, the leucines, and proline.

Lysine, on the other hand, was consistently present in the globulins though absent from zein, and the glycine content of the three globulins was higher than that of zein. The serine, threonine, and valine content was almost the same in all these four plant proteins.

The general conclusion, therefore, is that the quantitative paper chromatographic method can now be applied to the analysis of the amino-acid composition of the protein fraction of plants, even if they are only available in small amount.

II. A COMPARISON OF THE COMPOSITION OF THE ALCOHOL-SOLUBLE AND ALCOHOL-INSOLUBLE NITROGEN FRACTIONS OF THE POTATO TUBER

Only rarely in physiological work will it be possible to separate the insoluble-nitrogen fraction into its component pure proteins. Consequently it will be necessary to analyse what may be a mixture of proteins. As an example of the application of this quantitative method to an alcohol-insoluble nitrogen fraction, the analyses of the hydrolysate of an alcohol-insoluble acid-soluble fraction of the potato tuber (variety 'Sebago') are given in Table VI. These figures are to be compared with those for the alcohol-soluble nitrogen fraction in the same table. In this table the percentage of nitrogen in each compound in the soluble-nitrogen fraction has been calculated both including and excluding the amides. This was done because about three-fourths of the soluble-nitrogen in this variety was in the form of amides. A more convenient comparison of the relative amounts of the amino-acids in the soluble and insoluble fractions is thus obtained by excluding the amides from the calculations.

Before comparing the results obtained in the soluble and insoluble fractions certain general comments may be made. It is clear that varietal differences in the composition of potato tubers exist. Earlier analyses (Steward and Preston, 1940) of the familiar British variety 'King Edward' consistently showed that approximately two-thirds of the total nitrogen was alcohol-soluble and, of this, approximately one-quarter was in the form of amide-N, or approximately one-half of the soluble-N was accountable as asparagine and glutamine. Asparagine was in excess of glutamine in the approximate ratio of 3:2.

The composition of the newer U.S. variety 'Sebago' is somewhat different, and it is also quite evidently different in the analysis of its soluble compounds from the variety 'Katahdin', as previously reported by the authors (Steward

et al., 1949). From the limited data available the main points would seem to be as follows:

(*a*) The variety 'Sebago' contains approximately the same amount of total nitrogen (2·14 mg. N per g. fresh wt.) as the variety 'King Edward' (2·06 mg. N per g. fresh wt.), but the tubers of the variety 'Sebago' were relatively richer in protein (0·9 mg. N per g. fresh wt.) than the 'King Edward' (0·68 mg. N per g. fresh wt.) and consequently poorer in soluble nitrogen.

(*b*) By contrast the variety 'Katahdin' is richer in total nitrogen (2·52 mg. N per g. fresh wt.) and the increase is predominantly in the soluble fraction.

(*c*) Whereas glutamine and asparagine together account for about 50 per cent. of the soluble nitrogen of the variety 'King Edward', they account for nearly 75 per cent. of the somewhat smaller soluble fraction in 'Sebago'. The amide content of 'Sebago' is, however, not only a relatively greater part of the total soluble nitrogen fraction, it is also somewhat greater on the absolute basis (0·87 mg. N per g. fresh wt. present as asparagine and glutamine, compared with 0·70 mg. N per gm. fresh wt. in 'King Edward'). By contrast, however, the variety 'Katahdin' has an even greater total content of soluble nitrogen in the form of the two amides (1·03 mg. N per g. fresh wt.).

(*d*) Whereas asparagine was the predominant amide of the tubers used by Steward and Preston (1940), the two amides of 'Katahdin' were present in almost equal amounts. 'Sebago' is distinguished by the fact that the content of glutamine actually exceeded that of asparagine in the approximate ratio of 2:1, which is a higher ratio than any recorded by Neuberger and Sanger (1942) and is the highest encountered in our experience.

These facts are mentioned here to show that the new methods of quantitative partition chromatography on paper reveal that the alcohol-soluble fraction of the potato is not constant in its detailed composition but that differences which may be genetic or due to cultural and or climatic conditions during growth do occur. It is, however, of interest to compare the relative composition of the alcohol-soluble nitrogen fraction and that of the acid hydrolysate of the insoluble (protein) moiety from the same tubers.

The data of Table VI clearly show that there is no necessary correlation between the nitrogen compounds which make up the composition of the soluble and insoluble fractions respectively. This should dispose of any theories based upon the oversimplified idea that the soluble constituents are the immediate precursors of protein and that their concentrations determine rates of synthesis. (Free aspartic acid, free glutamic acid, glycine, lysine, proline, leucines, and phenylalanine are relatively more prominent in the insoluble fraction, whereas arginine, methionine, valine, and γ-aminobutyric acid are more abundant in the soluble fraction.) The relative abundance of γ-aminobutyric acid in the soluble fraction and its complete absence from the protein as well as the relative abundance of proline in the protein and its almost complete absence in the free state are evidence enough of this important fact. γ-Aminobutyric acid constitutes 11 per cent. of the soluble nitrogen and it is the fourth most important constituent. This observation

re-emphasizes that only the α-amino (or imino) acids are to be found in the proteins. The failure of the tissue to combine the relatively abundant γ-amino group as such into its protein must have a meaning for the mechanism of synthesis when this is understood.

Table VI

Composition of the alcohol-(80%) soluble and alcohol-insoluble fraction of the potato tuber (var. 'Sebago')

The potato tuber was thoroughly ground with enough 95% alcohol to make the final alcohol concentration 80%. This was centrifuged and the residue was re-extracted three times with 80% alcohol, then the residue was extracted with hot 80% alcohol and all alcohol extracts were combined and analysed.

The residue was hydrolysed with 4N HCl for 20 hours at 115° C. The insoluble material was separated and washed with 4N HCl and the acid-soluble fraction was analysed. The ammonia was determined by the method of Pucher *et al.* (1935).

96·3% and 87·5% of the total Kjeldahl nitrogen in these samples is accounted for by these analyses of the soluble and insoluble nitrogen fractions.

	1	2	3	4	5	6	7
	Alcohol-soluble fraction				Alcohol-insoluble fraction		
Amino compound	μg./g.	μg.N/g.	% of N	% of N excluding the amides	μg./g. fresh wt.	μg.N/g. fresh wt.	% of N
Aspartic acid	106·6	11·22	0·91	3·09	708	72·4	7·92
Glutamic acid	178·2	16·95	1·37	4·67	837	79·7	8·71
Serine	66·2	8·81	0·72	2·42	213	28·45	3·11
Glycine	27·6	5·15	0·42	1·42	319	59·8	6·54
Asparagine	1,374·0	291·5	23·6	—	—	—	—
Threonine	100·7	11·83	0·96	3·26	176	20·7	2·26
Alanine	132·0	20·75	1·68	5·72	353	55·5	6·06
Glutamine	3,020·0	579·0	46·9	—	—	—	—
Lysine	63·2	12·1	0·98	3·33	458	87·7	9·59
Arginine	356·2	114·5	9·29	31·5	341	109·7	12·0
Methionine	82·8	7·74	0·63	2·13	95·4	8·1	0·89
Proline	trace*	trace	trace	small	201	24·5	2·68
Valine	243·4	29·1	2·36	8·02	450	53·8	5·88
Leucines	104·4	11·15	0·90	3·07	1,005	107·2	11·74
Phenylalanine	138·0	11·71	0·95	3·22	726	61·6	6·73
Tyrosine	121·4	9·38	0·76	2·58	230	17·26	1·89
γ-aminobutyric acid	299·8	40·75	3·30	11·22	0·0	0·0	0·0
Ammonia	63·2	52·1	4·22	14·3	155·3	128	14·00
Total	6,414·5	1,233·7	99·95	99·95	6,267·7	914·4	100·0

* Less than 15 μg. per g., which corresponds to less than 4 μg. per chromatogram.

Although it is clearly not possible to say how much combined asparagine and glutamine exists in the protein, it is clear that this amount can in no way reflect the abundance of these substances in the soluble fraction. At a maximum 28 per cent. of the nitrogen of the insoluble fraction could be combined as amides, assuming that *all* of the ammonia in the hydrolysate came from amides. However, asparagine and glutamine together accounted for over 75 per cent. of the nitrogen of the soluble fraction in this tissue. There is

also no necessary proportionality between the different amino-acids as they exist in the free state and as they occur in the protein, indicating that the relative proportions of the free amino-acids in no way reflect their frequency of combination in the proteins. These facts are clearly more compatible with the view that the alcohol-soluble nitrogen compounds of the potato tuber represent a pool of nitrogen compounds from which nitrogen is withdrawn when synthesis occurs and that little or no significance is to be attached to the amino-acids which occur free as the direct intermediates of protein synthesis.

The following comparisons between the soluble and insoluble nitrogen fractions of the potato tuber may now be summarized.

1. It is, of course, impossible to detect combined amides in the insoluble fraction. However, it is obvious from the quantities of aspartic acid, glutamic acid, and ammonia that the amides are not as preponderant a constituent of the insoluble fraction as they are of the soluble fraction. At a maximum, 28 per cent. of the nitrogen of the insoluble fraction could be combined as amides (assuming all the ammonia came from amides), whereas asparagine and glutamine account for 78 per cent. of the nitrogen of the soluble fraction.

2. γ-aminobutyric acid does not occur combined in the insoluble fraction, whereas it contributed 11 per cent. of the non-amide nitrogen of the soluble fraction and is the fourth most important constituent of the soluble nitrogen. This observation re-emphasizes that only the α-amino- (or imino) acids are to be found in proteins. The failure of the tissue to combine the relatively abundant γ-aminobutyric acid in its protein must have a meaning for the mechanism of protein synthesis which cannot use the γ-amino group directly.

3. In the potato tuber proline occurs in the protein fraction but is not present in quantity in the free state. Proline has been detected free in the potato tuber (Dent, Stepka, and Steward, 1947) and usually can be detected if sufficient care is taken.

4. Comparing the relative importance of the amino-acids (cf. Table VI, columns 4 and 7), it is seen that glycine, lysine, proline, leucines, and phenylalanine are relatively more prominent in the insoluble fraction, while arginine, methionine, valine, and γ-aminobutyric acids are relatively more abundant in the soluble fraction. The latter acids may be more active metabolically than the other amino-acids.

The relatively high content of aspartic and glutamic acids in the insoluble fraction (as shown in columns 5 and 6 of Table VI) may be misleading when compared with the soluble fraction (columns 1 and 2). This is so because it has not been possible to determine the amount of aspartic and glutamic acids present in the proteins as asparagine and glutamine respectively. If one assumes, as may well be true, that the ammonia in the hydrolysates of the insoluble fraction derives mainly from the amides, then it is apparent that 84 per cent. of the aspartic and glutamic acids could be accounted for in the proteins as combined amides. Thus in the insoluble fraction as in the soluble fraction most of the aspartic and glutamic acids would be present as their amides. On this basis asparagine and glutamine would still appear as out-

standing examples of substances that are relatively more abundant free in the soluble fraction than combined in the insoluble fraction, and the contribution of aspartic and glutamic acids would also seem to be greater to the soluble-N than to the insoluble-N fraction.

Neuberger and Sanger (1942) have shown that nearly 90 per cent. of the protein of the potato tuber is soluble in 2 per cent. NaCl, and this is called 'tuberin'.

TABLE VII

Comparison of the chromatographic analyses of the insoluble nitrogen fraction of potato with other analyses of potato tuber protein

Grams of amino-acid per 16 g. of N

Amino-acid	Alc.-insol. fraction by paper chromato-graphic method	Tuberin, Slack (1948)	Potato protein, Groot (1945)	Tuberin, Sjollema and Rinkes (1912)
Aspartic acid	12·0	—	—	—
Glutamic acid	14·6	—	—	4·6
Serine	3·7	—	—	—
Glycine	5·6	—	—	—
Threonine	3·2	5·9	6·9	—
Alanine	6·2	—	—	4·9
Histidine	—	2·2	2·2	2·3
Lysine	8·0	7·7	3·6	3·3
Arginine	6·0	6·0	4·8	4·2
Methionine	1·5	2·3	2·6	—
Proline	3·5	—	—	3·0
Valine	7·7	6·1	7·6	1·1
Leucine + *Iso*leucine	17·6	17·5	9·6 + 5·0 = 14·6	12·2
Phenylalanine	12·7	6·6	5·9	3·9
Tryptophane	—	1·6	2·3	—
Tyrosine	3·9	—	—	4·3
Cysteine	—	—	0·1	—
Cystine	—	2·1	0·8	4·4
Valine+alanine	—	—	—	8·2
Valine+leucine	—	—	—	1·9
Total	106·2	58·0	51·4	58·1

Table VII compares the analysis of the insoluble —N fraction of potato tuber and of tuberin as determined by several investigators. The results of Slack (1948) and Groot (1945) agree well except for lysine. The chromatographic value for lysine here reported agrees well with Slack's value. The chromatographic values agree generally with those of Slack except for threonine and phenylalanine. In addition, the chromatographic data extend the analysis of the protein by including values for aspartic acid, glutamic acid and serine, glycine and alanine. It should be noted that the analyses of potato tuber protein are similar to that of the seed globulin as reported in Tables I–V of this paper.

In conclusion, there is now at hand a method which can furnish a satisfactory account of the amino-acids present in the protein fraction of plants and this can be carried out upon samples containing as little as 50 μg. of

protein —N. There is, therefore, little reason why we should remain in ignorance of the detailed composition of the protein fraction of plants. Many otherwise baffling problems of development and morphogenesis may well be clarified when it is known how far the nature of the proteins which are synthesized can be regarded as determining the pattern and subsequent course of development or the extent to which the protein is itself the result of more deep-seated attributes of the regions in which its synthesis takes place.

ACKNOWLEDGEMENTS

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The Relation of Germination of Wheat to Water Potential[1]

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SUMMARY

The relation of moisture potential to the germination of wheat was studied by supplying water to the seeds in the vapour phase, at controlled relative humidities. An apparatus for this purpose and a method of attaining the necessary precise control of temperature are described.

The critical level of moisture potential at which germination is completely inhibited was not reached. Even when, as a result of infection, the duration of the experiments was limited to 20 days, up to 20 per cent. of the seeds germinated at a potential of −320 metres of water or a pF of 4·5. This is considerably drier than Permanent Wilting Point, which is represented by a potential of −160 metres of water, or pF 4·2. There is a marked effect of moisture potential on the time taken to germinate, as times of emergence varied from 2 to over 20 days over a range of potentials of from zero to −320 metres of water, but it is probable that very little further germination would have occurred after 15 days had not fungal infection enabled the seeds to obtain liquid water by bridging the air-gap between seed and water source. This infection is probably responsible for the reduction in viability of the seeds at the lower potentials, as there is an indication that under conditions where germination is postponed considerably, seeds exhibit a reduced germinating capacity probably due to an increased susceptibility to infection.

The failure of some individual seeds to germinate at potentials which allow other seeds to germinate is not due to failure to attain a critical moisture content.

In soil, the percentage germination at low potentials is similar to that observed without soil, but the reduction in the total germinating capacity, or viability, after 20 days is not as great, and this is probably due to the reduced incidence of visible infection observed under these conditions.

INTRODUCTION

THE rate at which water is taken up by a seed after it has been sown in the soil must be determined partly by properties of the seed and also by the magnitude of the forces tending to retain water in the soil. The aggregate of these forces is often termed the soil suction. Buckingham (1907) called it 'capillary potential' and defined it as the height in centimetres of the water column equivalent to the soil suction. Alternatively (Schofield, 1935), it may be expressed on the pF scale, where pF is the logarithm to base 10 of the capillary potential. Schofield pointed out that the term 'capillary potential' is objectionable, because it suggests surface-tension effects, and, at a discussion on water movement in plants held by the Faraday Society at Rothamsted in 1949, proposed the alternative term 'water potential'. The water potential

[1] The work described in this paper formed part of a thesis for the degree of Ph.D. in the University of London.

at a given point in the soil is a measure of the work required to move unit mass of water from that point to a free water surface, that is, it is a difference in free energy. The great virtue of the water potential concept is that, being an energy relationship, its definition is independent of the particular mechanisms involved, whether they be osmotic effects due to the presence of solutes in the soil water, surface effects in water films on the soil particles, or colloidal effects associated with the clay fraction of the soil. It may therefore be applied to other systems than the soil, including plant tissues, and the movement of water from the soil through the plant to the atmosphere may be regarded as the consequence of a gradient in water potential along the path of movement (e.g. see Weatherly, 1951). As the potential of free water is taken as zero, the water potential in unsaturated soil must have negative values, increasing numerically with decreasing moisture content. It is convenient to express water potential, d, in metres of water rather than centimetres, and on this basis pF is equal to $\log_{10} d+2$.

The object of the work described in this paper was to determine how germination of wheat seeds depends on water potential, and in a later paper the effect of varying water potential in the environment of a seed on the rate of water uptake by the seed will be considered.

Several workers have studied the dependence of germination on soil moisture content. Peters (1920) and Doneen and MacGillivray (1943), for example, have shown that seeds will germinate in soil with a moisture content at or below the Permanent Wilting Point ($d = -160$ metres, pF = 4·2 approximately). Seeds germinated in a shorter time at high soil moisture content than at low. The ability to germinate at low soil moisture contents did not seem to be correlated with size of seed.

It is difficult to hold the water potential in soil at prescribed values, and for precise experiments on the effect of varying water potential it is necessary to use simpler systems. One possible method is to make use of solutions of varying concentrations. Thus Uhvits (1946) germinated alfalfa seeds on filter-paper wetted with mannitol or sodium chloride solutions, and found that germination, in a period of about 10 days, was inhibited by solutions having osmotic potentials of 12–14 atmospheres ($d = -120$ to -140 metres). This method, involving contact between seeds and solutions, is open to the criticism that the seeds may absorb the solute as well as water, so that the water potential of the seed may be changed, and the difference in water potential between solution and seed altered, independently of the change produced by water movement into the seed. Further, as Ayers and Hayward (1948) have pointed out, accumulation of solute in the seed may have toxic effects, reducing the percentage germination and decreasing the rate of germination of the seeds that remain viable, and so obscuring the direct effect of varying water potential on germination. Uhvits found that sodium chloride solutions had a greater retarding effect on germination than mannitol solutions of equal osmotic potential, suggesting that the sodium or chloride ions had toxic effects on the seeds.

In the present work any possibility of complications from solute uptake was avoided by supplying water to the seed in the vapour phase. Instead of immersing the seeds in solutions of varying concentration they were held in the atmosphere of a confined space above the solution, so that the water potential in the environment of the seed was controlled by varying the relative humidity. When equilibrium exists between an aqueous solution and water vapour, the potential is the same in both phases. At room temperature, water vapour is sufficiently rarefied to obey the ideal gas law: consequently vapour at a pressure (or partial pressure) p has a potential lower than that of the saturated pressure (or partial pressure) p_0 by $RT \log_e \frac{p_0}{p}$.

Very precise measurements of the aqueous vapour pressure of pure water and of solutions of sodium chloride are on record, and from these the lowering of potential of water due to each concentration of sodium chloride can be calculated as shown later.

EXPERIMENTAL METHODS

Plant material. The seeds[1] chosen for study were of wheat var. 'Squareheads Master 13/4', harvested in 1948, obtained from the National Institute of Agricultural Botany. Wheat is known to maintain a fairly constant germinating capacity for quite long periods, and the seeds are of convenient size and shape.

After individual weighings had been made of about 200 seeds on a torsion balance, it was decided to use seeds whose weights lay between 40 and 60 mg. This group contained about 80 per cent. of the sample and provided seeds of a standard size that fitted easily into the wire cradles. All seeds were treated with an organo-mercurial dressing to reduce fungal infection.

Apparatus. It was desired to maintain a constant known water vapour pressure in the gas phase at the surface of the seed. To achieve this the following conditions had to be satisfied:

1. The diffusion path of water vapour between the surface of the control solution and the seed should be as short as possible, so that the fall in water potential across the air-gap should be negligible, and the potential at the seed surface not sensibly different from that at the surface of the solution.
2. The vessels containing control solution and seeds should be held at accurately controlled constant temperature. It was especially necessary to avoid rapid temperature fluctuations as these would set up temperature gradients within the system, which might lead to distillation from the solution to the seed and the condensation of liquid water on the seed surface.

Controlled humidity chambers. These consisted of glass sample tubes 3 in. long by 1 in. diameter closed by rubber bungs, each bearing a capillary tube 12 cm. long and 1 mm. bore to allow pressure equilibration with the external

[1] The term 'seed' has been used to denote the wheat caryopsis which is functionally a seed.

atmosphere when the tubes were placed in the thermostat at a temperature different from the laboratory temperature. It was calculated that the rate of diffusion of water vapour through the capillary tube was about 0·5 mg. per week. This quantity was negligibly small compared with the amounts of water absorbed by the seeds, and was insufficient to cause a significant change in the concentration of the solutions. Each sample tube contained five hollow paper cylinders made by rolling strips of No. 1 Whatman filter-paper on glass rod formers 6 mm. in diameter, and fixed with cellophane tape. The wheat grains, previously sorted to reject extremes of weight, were of average dimensions 7×4 mm. so that, when suspended within the ends of the paper tubes with their long axes upright, there was an air-gap of about 1 mm. between the grain and the internal walls of the paper tube. The grains were held within the paper cylinders by means of supporting cradles made from Assay Silver Wire No. 20 S.W.G. (0·036 in. diameter), soldered at the joints with pure tin. Silver wire was chosen to minimize any possible effect of metal toxicity, and because of its high thermal conductivity.

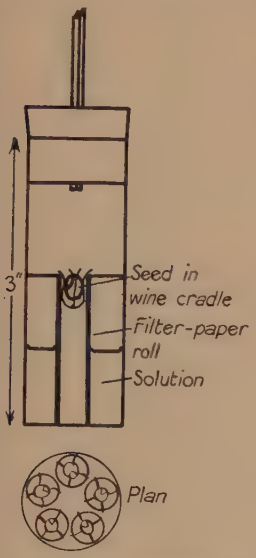

FIG. 1. Controlled Humidity Tube

When the sample tube contained about 5 ml. of solution the paper tubes rapidly became saturated and provided a uniformly wet surface at a fairly uniform distance from the greater part of the seed surface (see Fig. 1, where, in longitudinal section, only one paper tube is shown). No evidence of capillary 'creep' of solution along the silver wire was found, even over periods of 2–3 weeks.

As the seeds were respiring, the possibility of a high level of carbon-dioxide concentration being attained within the tube was considered. Barnell (1937) gives data for production of carbon dioxide by barley seeds, and if that for wheat is assumed to be of the same order, the five seeds per tube should produce about 5 mg. per week at the time of emergence so that if the seeds remained in the tubes for 1 week after emergence the carbon dioxide concentration could reach about 8 per cent. As, however, the amount of carbon dioxide produced before emergence is very much less, and the seeds were usually removed soon after emergence, and as CO_2 could diffuse through the open capillary tube, it is unlikely that any such level was reached.

Temperature control. The accurate maintenance of a desired relative humidity in the system called for a very precise control of temperature. A temperature change of 0·001° C. within the tubes would cause a change of water potential of approximately 0·8 metres of water, and any rapid fluctuations of temperature of an order greater than this could produce temperature gradients between the surface of the seeds and the paper tubes, and lead to condensation on the seeds.

The characteristics of the toluene-mercury thermoregulator make it unsuitable for use in its usual form, as the temperature fluctuations when the control system is cycling are too large. More efficient control is possible where the level of mercury in a thermoregulator decides the proportion of 'off' to 'on' time of the heaters, rather than whether they should or should not be heating.

Trials with such a 'proportioning head' on a toluene-mercury thermoregulator of standard type gave good control of short-period changes, but

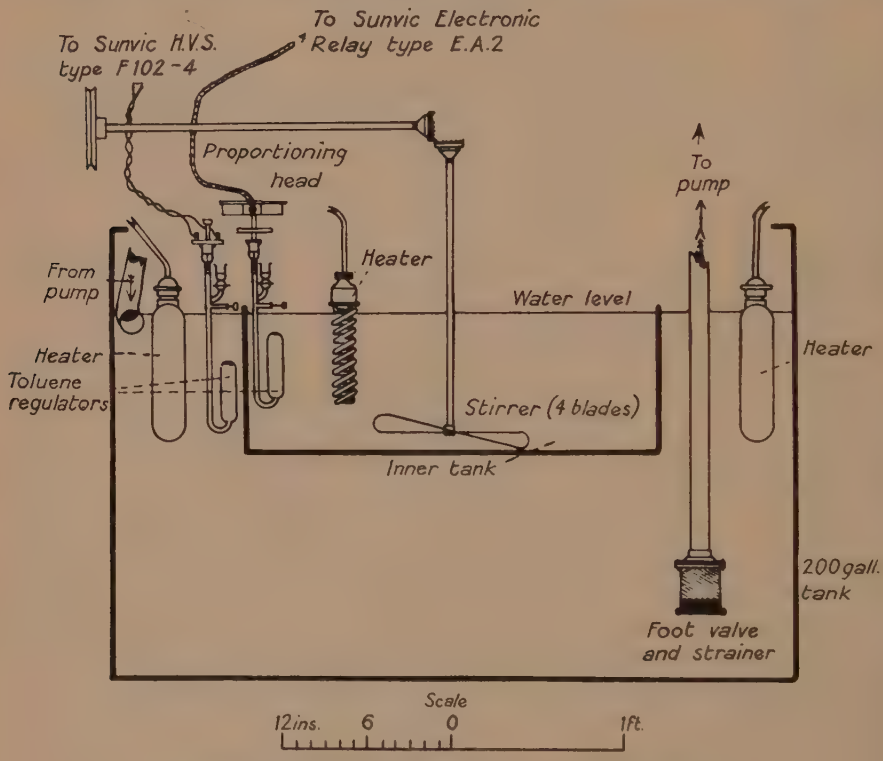

FIG. 2. Thermostat Equipment

the effects of long-period changes in room temperature were not entirely suppressed.

The final design made use of two-stage control. A 200-gallon domestic water tank was equipped with a conventional type toluene-mercury thermoregulator controlling, through a hot-wire vacuum switch, three 250-watt heaters. An electric pump, of capacity 300 gallons per hour, circulated the water in this tank, and the control obtained was better than $\pm 0{\cdot}1°$ C. The large volume of water was an advantage in coping with the usual fluctuations in room temperature.

Within this tank a smaller tank 30 in. × 20 in. × 10 in. was sunk almost to its rim and suspended rigidly. The control in this tank was effected by a toluene-mercury thermoregulator, bearing a proportioning head, controlling the heater through a thyratron type electronic relay. The heater was in the form of a helix, with the minimum amount of insulation between heating wire and casing tube so as to provide the minimum heat capacity and the maximum heating surface. The bath was stirred with a large four-bladed

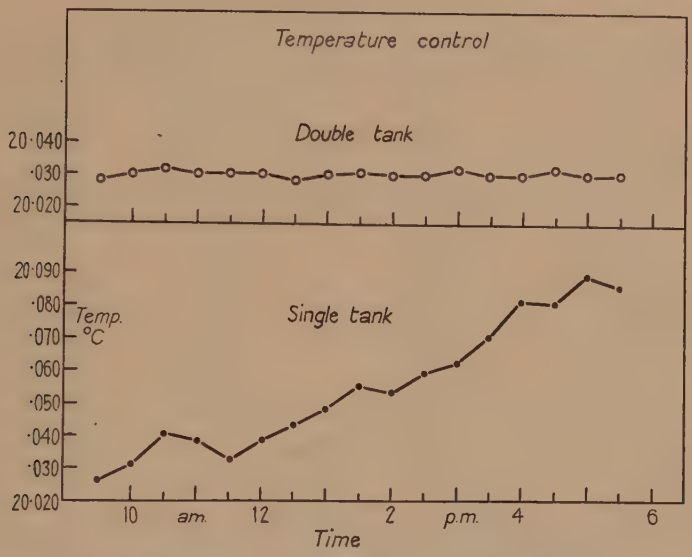

FIG. 3. Temperature changes observed in thermostats

propeller. A sectional view is shown to scale in Fig. 2. The inner bath was large enough to hold 80 sample tubes.

Tests with a Beckmann thermometer confirmed the efficiency of the stirring in the inner tank and provided the performance data shown in Fig. 3. The upper points demonstrate the efficiency of the double tank with both control circuits working.

Calculation of water potential. The potential was controlled by the use of solutions of sodium chloride. Data on the relation of vapour pressure lowering to molar-concentration of the salt solution was provided by Mr. G. H. Cashen (Soil Physics Department, Rothamsted) in the form of values of proportional vapour pressure lowering $\left(\frac{P_0-P}{P_0}\right)$ for the range of concentrations used. These are given in Table I. The equivalent values of the moisture potential, as given in column 3 of the table, were calculated from the formula:

$$d = \frac{RT}{100gM}\left(-\log_e \frac{P}{P_0}\right),$$

where d = moisture potential in metres of water,

$R = 8{\cdot}314 \times 10^7$ ergs, per °C., per mole,

T = absolute temperature,

$g = 980{\cdot}616$ dynes per gram,

$M = 18{\cdot}016$ (mol. wt. H_2O).

TABLE I

The relation of concentration of NaCl *to vapour pressure lowering at 20° C. and moisture potential*

Molar conc. NaCl	$\frac{P_0-P}{P_0}$	Potential, d (metres of water)	pF
0·20	0·00656	−90·8	3·95
0·25	0·00816	−113·0	4·05
0·30	0·00976	−135·2	4·13
0·35	0·01152	−159·8	4·20
0·40	0·01316	−182·7	4·26
0·45	0·01480	−205·4	4·31
0·50	0·01644	−228·7	4·36
0·55	0·01809	−251·8	4·39
0·60	0·01974	−275·0	4·44
0·65	0·02140	−298·3	4·47
0·70	0·02306	−321·6	4·51
0·75	0·02473	−344·9	4·54
0·80	0·02640	−369·1	4·57

The pF in column 4 of Table I is the logarithm of the values of d in cm. The relationships between concentration of sodium chloride, relative humidity, moisture potential, and pF at 20° C. are shown in Fig. 4.

EXPERIMENTAL PROCEDURE

The first part of the investigation consisted of an attempt to determine whether there was a critical level of water potential, of an order likely to occur under natural soil conditions, below which seeds are unable to germinate. The first sign of emergence of the primary root from the embryo was taken as the criterion of germination and in order that this could be readily observed the seeds were always placed with the embryo upwards.

Preliminary determinations of percentage germination were carried out on seeds placed on moist filter-paper, and, in all cases, germination was 99–100 per cent. Seeds were then placed in cradles in the controlled humidity tubes over distilled water to determine how quickly they would germinate in a saturated atmosphere, i.e. at zero potential. Germination occurred after about 48 hours at 20° C.

After tests with different concentrations of sodium chloride it was decided to work with concentrations of 0·25 M. to 0·70 M., representing water potentials of −113 to −321 metres of water. It was found that after a period

of about 20 days serious fungal or bacterial infection developed, in spite of the mercurial seed dressing. Surface sterilization of the seeds was difficult, as any immersion treatment was inadvisable, and although various vapour treatments were tried, no better alternative to the organic mercurial dust was found, and it was therefore decided to limit the duration of the experiments to 20 days. At the end of this time the seeds were often badly infected and the gaps between seeds and paper tubes were sometimes bridged by hyphae.

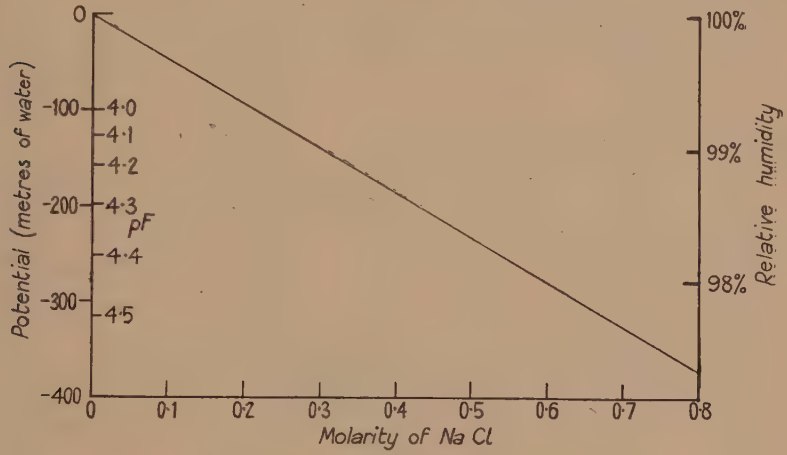

FIG. 4. Relation of water potential, relative humidity and pF to concentration of sodium chloride at 20° C.

It is known that fungi are usually present beneath the epidermis of the pericarp of wheat grains (Hyde, 1950), and this presumably accounts for the failure of the sterilization treatments.

To determine whether seeds that had failed to germinate at the end of the experimental period were still viable, they were transferred to moist filter-paper in an incubator at 20° C. and the number finally germinating in these conditions was recorded. The total percentage germination arrived at in this way was lower than the percentage germination of samples put directly from storage on to wet filter-paper. Fig. 5 shows that the loss of germination capacity tended to increase with increase in concentration of control solution; the regression coefficient of total germination percentage on concentration (in units of 0·01 M.) was $-0{\cdot}28 \pm 0{\cdot}14$, and just failed to reach significance at the 5 per cent. level ($t = 2{\cdot}06$ for $p = 0{\cdot}05$). Extrapolation of the regression line gives a value of 88 per cent. for germination at zero concentration. As the observed germination percentage at 20° C. on moist filter-paper was 99 per cent., this result suggests that the decline of viability with increasing concentration was more rapid in the range from 0 to 0·45 M. than in the higher concentration range shown in Fig. 5.

The cause of this loss of viability is not clear. Barton (1941, 1943) showed

that the germination capacity of seeds in storage decreased with increase in relative humidity in the range where germination was not possible. Transfer of the seeds from storage in laboratory air to the higher relative humidities may therefore account in part for the deterioration. Robertson *et al.* (1939) reported a decrease in percentage germination from 98 to 43 per cent. after 21 days at a relative humidity of 98 per cent. The tendency towards increased deterioration with decrease in relative humidity within the narrow range of humidity represented by Fig. 5 (98·5 to 97·7 per cent.) was probably a

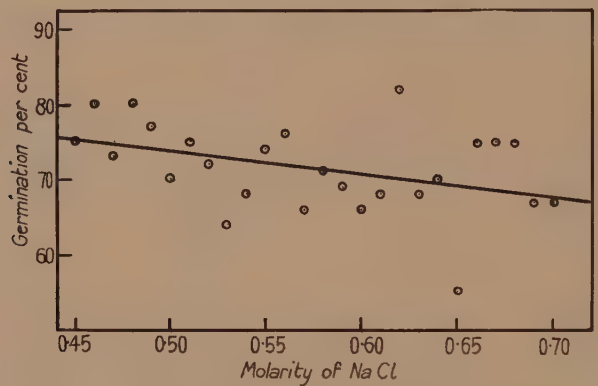

FIG. 5. Deterioration of total germination percentage at 20° C. with increased concentration

consequence of fungal or bacterial infection. Robertson reported mould development on all seeds at 79 per cent. R.H. or higher. Brewer and Butt (1950) found a more harmful effect of high humidities upon lupin seeds of low germinability than upon seeds of high germinability, and this supports the inference of Gilman and Semeniuk (1948) that, since the principal mould species found on seeds are saprophytes, deterioration caused by micro-organisms during storage is greatest in seeds initially low in vitality. Seeds already containing a large proportion of non-living material, therefore, are subject to continuing attack by saprophytic organism at humidities which are too low for mould development on more vigorous seeds. It seems likely that some seeds which would have germinated over the less concentrated solutions were rendered non-viable by the more intense infection that was able to develop in the longer germination time at the higher concentrations.

EXPERIMENTAL RESULTS

First series. Preliminary trials showed that water potentials corresponding to the Permanent Wilting Point did not inhibit nor greatly reduce germination. In subsequent experiments, therefore, progressively lower water potentials were included in the range tested, and eventually a range of concentrations from 0·45 M. to 0·70 M., corresponding to water potentials varying from —205 to —322 metres of water, was covered in steps of 0·01 M. The number

of seeds tested at each concentration was not constant but varied from 24 to 80. The pooled results of all the experiments are shown in Table II, which gives the number of seeds germinated, after 5, 10, 15, and 20 days, expressed both as a percentage of the total number of seeds tested ('uncorrected') and

TABLE II

Germination percentage at various moisture potentials (Series 1)

(A = uncorrected; B = corrected)

Conc. NaCl (M.)	5 days		10 days		15 days		20 days	
	A	B	A	B	A	B	A	B
0·45	57	77	65	87	73	97	87	100
0·46	57	72	75	94	80	100	87	100
0·47	55	76	70	97	70	97	70	97
0·48	55	69	70	88	75	94	78	97
0·49	45	58	67	87	73	94	75	97
0·50	33	47	52	75	59	85	62	91
0·51	28	38	47	62	62	83	62	83
0·52	16	22	47	65	58	80	69	85
0·53	21	33	47	74	53	83	62	87
0·54	37	53	52	75	57	84	79	86
0·55	28	38	47	64	54	74	71	88
0·56	21	38	46	61	52	69	75	88
0·57	11	19	31	47	38	57	50	79
0·58	10	14	39	54	45	63	72	91
0·59	6	9	31	45	36	53	66	91
0·60	1	9	24	36	28	41	62	94
0·61	12	18	27	41	28	41	46	70
0·62	5	6	32	39	38	45	75	82
0·63	0	0	30	44	35	52	62	93
0·64	2	4	25	36	40	57	58	82
0·65	2	5	27	34	40	50	47	69
0·66	0	0	25	33	37	50	38	50
0·67	4	5	25	33	25	33	33	44
0·68	0	0	29	39	37	50	42	55
0·69	4	5	21	31	21	31	25	37
0·70	0	0	8	12	12	19	12	19

as a percentage of total viable seeds, determined as the sum of those germinating during the experimental period or subsequently on moist filter-paper ('corrected').

In the following discussion only the corrected values will be considered, since the correction eliminates differences due to variation in initial viability, and possibly also in susceptibility to microbial infection, if the explanation given above for the decline in viability during the experimental period is correct.

It is evident from Table II that the marked fluctuations in uncorrected germination percentage from a smooth relation to salt concentration persisted after correction for viability. It follows that the main cause of the variability between samples, to which the fluctuations are attributable, was not variation in the proportion of viable seeds, but differences in the reaction of viable seeds to change of moisture potential.

The similarity of the fluctuations at the different observation times, e.g. the consistently low germination percentage observed for salt concentration of 0·051 M. and 0·052 M., compared with higher and lower concentrations, is a consequence of the fact that the same seeds were observed at all times, so that the results for the four observation times were not independent.

The data relating percentage germination to salt concentration were smoothed by fitting polynomial curves. It was found that for the first three times of observation quadratic curves gave an adequate fit; none of the cubic regressions were significant. The regression coefficients of the orthogonal polynomials for these times were:

Days	Linear	Quadratic
5	$-1{\cdot}58\pm0{\cdot}105$	$0{\cdot}298\pm0{\cdot}062$
10	$-1{\cdot}43\pm0{\cdot}091$	$0{\cdot}089\pm0{\cdot}054$
15	$-1{\cdot}44\pm0{\cdot}101$	$0{\cdot}047\pm0{\cdot}061$

All the linear coefficients were highly significant and of similar magnitude. The quadratic coefficient for day 5 was also significant, but that for day 10 was smaller and did not greatly exceed its standard error. At day 15 there was no evidence of a departure from a straight line relation.

The regression equations in terms of x, the salt concentration measured in units of 0·01 M., calculated from the orthogonal polynomials, were:

Day 5 . . $y = 693-20{\cdot}3x+0{\cdot}149x^2$

Day 10 . . $y = 365-\ 8{\cdot}0x+0{\cdot}045x^2$

Day 15 . . $y = 230-\ 2{\cdot}9x,$

where y = corrected germination percentage. These curves are plotted in Fig. 6. They indicate that after 4 days nearly 80 per cent. of viable seeds had germinated over 0·45 M. salt solution, and the germination decreased, at first rapidly and then more slowly, with increase in concentration. Less than 5 per cent. of seeds had germinated in the concentration range from 0·65 to 0·70 M. The increase in germination percentage between 5 and 10 days was greatest in the middle of the concentration range; it amounted to 35 per cent. for 0·60 M., compared with 15 per cent. for 0·45 M., and to 22 per cent. for 0·70 M. Similar changes were found between 10 and 15 days, and these account for the straightening of the curve and its eventual approximation to linearity. On the 15th day germination percentage fell from 100 per cent. at 0·45 M. to 29 per cent. at 0·70 M.

The smooth curve fitted to the data for the 20th day differed greatly from those for the earliest times of observation. It was found that the cubic term in the orthogonal polynomial was highly significant; the coefficients were:

Linear	Quadratic	Cubic
$-1{\cdot}11\pm0{\cdot}093$	$-0{\cdot}322\pm0{\cdot}056$	$-0{\cdot}0120\pm0{\cdot}0026$

The quartic term was also tested but was found not to be significant. The corresponding regression equation was $y = 3376 - 180x + 3{\cdot}30x^2 - 0{\cdot}0200x^3$; the curve it represents is also plotted in Fig. 6. It is evident that while there was little change in germination percentage between 15 and 20 days at both ends of the concentration range, in the middle of the range, for concentrations

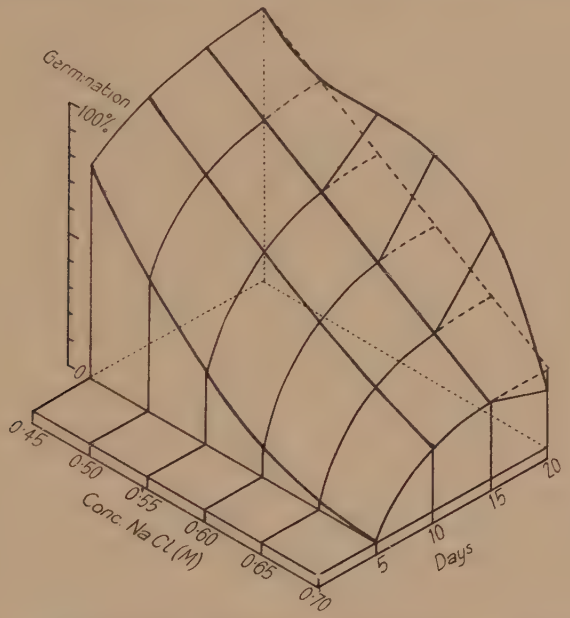

Fig. 6. Relation of time and solution concentration to germination (Polynomials fitted in one direction only.)

of 0·55–0·65 M., there was a more rapid increase than in the previous 5 days, so that for these concentrations the curves of increase in germination with time were sharply inflected upwards. The upward bulge in the curve for the 20th day in the middle of the concentration range accounts for its cubic form.

The change of form in the curve between the 15th and the 20th day is considered to be a consequence of the growth of fungus mycelium bridging the air-gap between the seed and the paper cylinder, which led to a breakdown of the control of water potential and brought the seeds into direct contact with the solution. This could not increase the germination percentage for concentrations near to 0·45 M., as nearly all viable seeds had already germinated by the 15th day, but in the range from 0·50 M. to 0·60 M. it produced a progressively greater increase in germination as the percentage of viable but ungerminated seeds increased. The decline in the effect of the bridging of the gap with increase in concentration 0·60 M. to 0·70 M. must be ascribed to some other effect, and a possible explanation is that at water potentials represented by these concentrations the growth rate of the fungal

mycelium was reduced so that at 0·70 M. the bridging of the air-gap was ineffective. It is therefore assumed that, in the absence of fungal infection, the relation between percentage germination and salt concentration would be similar to that shown by the broken line in Fig. 6 obtained by smooth extrapolation of the progress curves of germination with time for different concentrations. This indicates a linear fall of germination percentage from nearly 100 per cent. at 0·45 M. to approximately 30 per cent. at 0·70 M.

TABLE III

Water content, per cent. of dry matter of germinated (G) and ungerminated (U) seeds (Series 2)

(Mean water content of 60 seeds at each time for each concentration)

	0·55 M.				0·65 M.				0·75 M.			
	Seeds germinated		Water content		Seeds germinated		Water content		Seeds germinated		Water content	
Time (days)	No.	%	G	U	No.	%	G	U	No.	%	G	U
5	29	48	50·4	48·7	7	12	51·1	45·8	0	0	—	44·5
10	52	87	53·0	50·2	31	52	50·7	49·2	6	10	46·2	48·1
15	53	88	58·0	51·2	32	53	51·3	48·9	13	22	48·1	47·3
20	55	92	65·6	61·0	38	63	61·1	56·0	10	17	54·8	56·0

If this explanation of the data for the 20th day is correct, it follows that germination at all water potentials studied was almost completed by 15 days. Salt concentrations between 0 and 0·45 M. representing a range of water potential from 0 to −205 metres had no detectable effect on the final germination percentage, though the rate of germination decreased with decrease in water potential. At still lower water potentials the final percentage germination as well as the rate of germination was decreased, but 30 per cent. of seeds were capable of germination at a potential of −320 metres.

Second series. To determine whether the failure to germinate of some of the seeds at the higher concentrations was due to inability to attain some critical moisture content further experiments were carried out. Seeds were placed over solutions of 0·55 M., 0·65 M., and 0·75 M. and at intervals of 5 days samples were removed, the seeds sorted into germinated and ungerminated groups, and the moisture contents of each group determined by oven-drying. The results are shown in Table III.

The number of seeds in each group were recorded, and, although these experiments differed from those of Series 1 in that the same seeds could not be observed at all times, and that no 'corrected' germination counts could be obtained, the general trend confirmed the results of Series 1. The numbers of seeds are out of a possible total of 60 for each observation.

The table shows that, although the moisture contents of the germinated seeds were fairly consistently higher than those of the ungerminated seeds, failure to germinate could not be attributed wholly to reduced ability to

absorb water. This is clear from the fact that, at 20 days over 0·55 M., for instance, the non-germinated seeds have a moisture content of 61 per cent., whereas at 5 days at this concentration the germinated seeds had a much lower moisture content, i.e. 50·4 per cent. This behaviour confirms the suggestion in Series 1 that fluctuations in germination percentage are due to differences in the reaction of viable seeds held at the same moisture potential and not to failure to attain a critical moisture content. The differences between the moisture contents of the two groups of seeds become smaller and irregular at the highest concentration, where, presumably, the selective effect of very low potentials upon a given sample of seed becomes more marked.

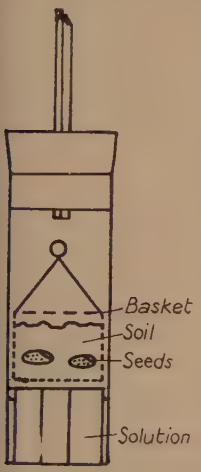

Fig. 7. Controlled humidity tube for soil

Third series. The irregular germination of the grain at low potentials and the considerable growth of fungi observed prompted consideration of the possible behaviour of the grain in soil under similar extreme conditions. Accordingly, comparative tests were made at a potential of −320 metres, with grain buried in soil.

Small quantities of a clay soil were suspended in wire gauze baskets within the sample tubes, the bottoms of the baskets being separated from the tops of the filter-paper rolls by an air-gap of a few millimetres (see Fig. 7). The soil had been sieved and fraction 0·5–2·0 mm. retained for use; one portion of this fraction was heated at 105° C. for 24 hours, while the remainder was air-dried. Samples of both soils were suspended in the baskets over 0·70 M. solution ($d = -321$ metres of water) and periodic weighings were made to follow the progress of the change in moisture contents and to check when equilibrium had been attained. After 100 hours both soils had attained equilibrium moisture contents of 6·3 per cent. for oven-dried and 8·4 per cent. for air-dried, the difference being probably due to changes in the physical structure caused by the heat treatment of the first soil.

Five seeds were then buried in the soil in each basket and the tubes left undisturbed in the constant temperature bath for 20 days. At the end of this time the amount of visible infection and the number of germinated seeds were noted. As in Series 1, the non-germinated seeds were then placed on moist filter-paper to determine the final number of viable seeds.

The amount of visible infection was considerably less than that observed on seeds in wire cradles in Series 1, but there was very little difference between the infection on seeds in the heat-treated and untreated soils. The corrected germination percentage was similar to that in Series 1, but the total number of viable seeds was greater. In both soils 80 per cent. of the seeds tested (30 per sample) retained their viability, as compared with 67 per cent. at a similar potential in Series 1. In both soil samples, when part of a seed was allowed to project above the soil surface, that portion almost invariably became infected.

DISCUSSION

Fig. 6 shows the marked effect of moisture potential upon the time taken for a seed to reach the stage of emergence of the radicle. The probable interpretation of the results of Series 1 and the possible causes of the deviation of the observed curve for 20 days from the general pattern of the other data has already been discussed in the presentation of the results, and it seems fair to deduce that the heavy infection occurring at the end of the experiment produced an anomalous result, probably mainly due to the limitations of the apparatus. Bridging of the air-gap between seed and paper tube may allow the seed to obtain liquid water or chloride solution, and this intake of salt may alter the osmotic properties of the seed and facilitate further entry of water into such infected seeds.

The critical level of moisture potential to inhibit germination completely is of a much lower order than was first expected. After 15 days almost 20 per cent. of the seeds germinated over 0·70 M. sodium chloride, representing a potential of −320 metres of water, a pF of 4·5, and a relative humidity of 97·69 per cent. Permanent Wilting Point is generally accepted to be at pF 4·2 or a potential of −160 metres of water. Under very extreme conditions, air-drying is capable of producing a soil pF of 6, or a relative humidity of as low as 50 per cent., but this would not normally occur in a temperate climate except at the very surface of the soil. As quoted previously, Jakobsen and Højendahl (1950) state, presumably of Danish soils, that the relative humidity in the soil rarely falls as low as 98 per cent. The conditions corresponding to a pF of 4·5 can, therefore, be considered as extremely dry.

Under the soil-moisture conditions of any fairly normal seed-bed there does not appear to be any reason, connected with water supply, why emergence of the radicle from the seed should not occur eventually, but this does not imply that subsequent satisfactory growth of the embryo will always occur.

The apparent deterioration in seed viability is a separate important factor. Fig. 5 shows that there is some indication that, after being subjected for some time to conditions under which germination is slowest, the germinating capacity of the seed is apparently decreased. This may be due, in the experiments described, to the fact that more of the seeds at the higher concentrations had been subjected to a prolonged period in the tubes before being transferred to moist filter-paper. The seeds germinating at lower concentrations were removed and thus did not have as high a probability of infection as those remaining in the tubes for longer periods. Potentials low enough to postpone seed germination for a considerable time do not, apparently, inhibit the growth of fungi, although there is evidence in the behaviour of the 20-day curve in Fig. 6 that potentials as low as −320 metres cause a decrease in the rate of growth of fungal hyphae.

Series 2 demonstrates that the failure of the proportion of the seeds to germinate in the time that other seeds at a similar potential have germinated is not due to any inability to attain a critical moisture content. Such a critical

point is apparently at a moisture content of less than 46 per cent. water on dry matter, and seeds after 20 days at a potential of −250 metres of water attained a moisture content of 61 per cent. without germinating.

Series 3, the comparison between seeds with and without soil, indicates that the germination percentage under the driest conditions studied is approximately the same in both cases. The reduced incidence of infection when the seeds were buried in soil suggested that the presence of soil reduced the growth of mycelium or spores present on or in the seed-coat. As heat-treated was as effective as air-dried soil, it is unlikely that this effect is due to soil organisms. The extra infection without soil seems to be responsible for the reduction in final germinating capacity.

ACKNOWLEDGEMENTS

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Further Observations on the Structure of Plant Cilia, by a Combination of Visual and Electron Microscopy

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WITH PLATES I–XI

SUMMARY

By combining visual microscopy of stained material with electron microscopy it has been possible to demonstrate fibrillar disintegration in the cilia of *Saprolegnia* (front and back), *Chlorosaccus* (front and back), *Allomyces*, and *Olpidium*. These represent three fungi and a yellow-green alga. All flagella showed eleven component strands, in most cases clearly differentiated into nine strands and a central pair. In addition, stained and unstained material of *Saprolegnia* and *Chlorosaccus* have been closely compared in the electron microscope and the comparison has included observations on one and the same cell with the electron microscope and the light microscope. The nature of the artifacts produced by staining has been investigated and the value of the combination of these various technical processes demonstrated. The phyletic importance of the hairs on the front flagellum in the yellow-green algae, brown algae, and water moulds is discussed.

INTRODUCTION

In a previous communication on the fungus *Saprolegnia* (Manton, Clarke, and Greenwood, 1951) a sample photograph of a stained zoospore taken at the highest power available to the light microscope was included and was referred to in the discussion in order to introduce a comparison between appearances in the light microscope and the electron microscope. The new observations to be presented below pursue this comparison in greater detail and in so doing have given considerable information, not only about details of ciliary construction which were difficult to elucidate by other means, but also about the nature of the artifacts which are produced by the staining process when stained material is examined with the electron microscope. Since both these types of information are of practical importance and collectively represent a new departure in the general techniques available to electron microscopists, it is convenient on this occasion to assemble results from a variety of different organisms in the context of a common technical approach, rather than to separate them out according to their taxonomic affinities as on previous occasions. This procedure has the additional advantage of revealing more clearly than would otherwise be possible the surprising degree of internal uniformity which prevails among plant cilia of very diverse types and origins.

As is well known, the staining methods devised by bacteriologists to show cilia are surprisingly powerful. Since the time of Loeffler (1889) it has been possible to detect, visually, objects such as bacterial cilia and the hairs on a *Flimmergeissel* which intrinsically are far below the limit of true resolution with the visual, or even the ultra-violet, microscope. It is true that these observations have not always been believed (for quotation of literature see Manton and Clarke, 1950); the matter for surprise is, however, not so much this as the fact that the observations could be made at all.

To elucidate this position further with the electron microscope seemed to us to require two types of observation, neither of which, so far as we know, has been attempted before, at least in plants. On the one hand we wished to be able to compare electron micrographs of stained and unstained examples of the same material. On the other hand we wished to amplify this by comparing if possible a visual photograph and an electron micrograph of one and the same stained cell. By these means we hoped to be able to evaluate with precision the exact identity of what it is which the light microscope is able to resolve after bacteriological staining, and to what extent this is dependent on alterations in the specimen.

This programme proved unexpectedly easy to carry out and the results obtained from it will be presented below separately for two organisms, the fungus *Saprolegnia* (p. 206) and the yellow-green alga *Chlorosaccus* (p. 208). Additional observations obtained incidentally by the new techniques will also be presented and these will be extended to two additional organisms, the fungi *Allomyces* and *Olpidium* (pp. 210–11). There will thus be three separate sections to the observational part of the paper and since the work for each has been carried out under somewhat different conditions, the names of the authors principally concerned will be separately enumerated at the beginning of each section.

MATERIAL AND METHODS

Bacteriological staining is normally applied to material which has been dried on a glass slide, either with or without previous fixation. This has been the starting-point for each of our four organisms which have all been killed by 30–60 seconds' exposure to osmic vapour, but not otherwise treated with chemicals.

After rinsing in distilled water to remove salts and the debris remaining behind on the glass after the first drying, the staining process adopted was exactly that described on p. 330 of Manton, Clarke, and Greenwood (1951). It consisted of a modification of Kirkpatrick's bacterial flagellum stain (cf. Mackie and McCartney, 1942, p. 213). This is a silver staining method following an iron tannin mordant. We have used the mordanting solution at stock strength and without the dilution quoted by Mackie and McCartney and for a rather longer time (15 minutes). In addition a temporary staining with aniline gentian violet has been superposed. This adds clarity to the preparation for purposes of visual photography, while still leaving only a faint pigmentation

in the ground. Since the gentian violet dissolves out almost at once in the subsequent handling, after the photography is finished, it is probable that it may be disregarded in the electron micrographs which express principally, if not entirely, the effect of the mordant and the silver staining only.

The visual photography was carried out in each case without a coverslip and with both specimen and lens immersed in Leitz 'Objectol'. This immersion fluid does not dissolve gentian violet and is easily removed with xylol. The lens used was a Cooke 1·8 mm. Fluorite objective (N.A. 1·3) and the source of illumination was a Pointolite lamp screened with green and orange (Wratten B and E) colour filters.

Transfer of photographed cells to the electron microscope carrier was achieved by means of a stripping film used in a manner recalling the procedure for ultra-violet microscopy (cf. Manton and Smiles, 1943). The stripping film, in this case formed from 2 per cent. nitrocellulose dissolved in amyl acetate, is poured on after the specimen has been passed through absolute alcohol and amyl acetate. Excess solution is then poured off and the slide allowed to dry. The relevant portion of the nitrocellulose film so formed is then floated off on to the surface of water and dried down again on the formvar film of a previously prepared electron microscope carrier. The nitrocellulose is then dissolved away and the specimen again allowed to dry. Shadow casting can then be applied in the usual way, the metal used in this case being 60:40 gold-palladium.

The electron microscope used is the Philips's instrument in the Botany Department of Leeds University. It should be noted that the magnifications which this gives are not quite constant on different occasions and with different accelerating voltages: the sizes given on the plates although approximately correct are therefore not exact.

SAPROLEGNIA

By I. Manton, B. Clarke, *and* A. D. Greenwood

The material used for this species (*S. ferax*) has already been described in detail (Manton, Clarke, and Greenwood, 1951) and therefore only the new observations need be given.

Fig. 1 shows two cells of the first motile stage stained as described above and photographed under oil immersion on the light microscope at a magnification of 2,000 diameters. They compare exactly with the second-stage cell published as Fig. 15 in our previous paper and, like it, show unmistakably the double row of hairs on the front flagellum and the short hind flagellum with its attenuated point.

Fig. 2 is an electron micrograph of part of these same two cells at a magnification sufficient to enable a precise comparison of the two views to be made, hair by hair. It is obvious that the light microscope can only just resolve a single hair, though a tuft of hairs is more clearly visible. The length of the hairs is thus fairly well indicated by the light microscope although their thickness is not.

Fig. 3 is a more enlarged part of the left-hand front flagellum of Figs. 1 and 2. If this is compared with Fig. 6 of the previous paper, the nature of the changes introduced by the staining are at once revealed. In the stained specimen (Fig. 3) a granular deposit has been laid down right through the specimen and in the ground, which obscures fine detail in both. The fine hair-points on the individual *Flimmer* hairs have become invisible or have been destroyed. The *Flimmer* hairs themselves have become greatly thickened, and this is no doubt the change of greatest importance in rendering them visible in the light microscope, as was surmised in another context by Manton and Clarke (1950).

Whilst these observations were being carried out for their own sakes, our attention was arrested by the detection on the stained preparations, when examined with the light microscope, of a few cases of fibrillar disintegration of flagella. These were very infrequent, being scattered amongst hundreds of normal cells and they would have been invisible to the eye without staining. Their scarcity was no doubt the reason why we had failed to find any when searching for them with the electron microscope on direct preparations of the ordinary kind, as explained in our previous paper. Once detected visually, however, they could be transferred to the electron microscope in the manner just described and at once the required observations could be made.

Figs. 4 and 5 show parts of the front and hind flagella belonging to one cell. In Fig. 4 the numerous detached *Flimmer* hairs show at once that this is the front flagellum. The axis sheath has vanished, as is usual in such cases, but the axis itself had dismembered very clearly into eleven strands, two of which are central. The central strands take a more undulated course than the others after dismemberment and drying, and they are more easily broken; they must therefore be of a different physical texture. We have not been able to determine their relative length owing to the fact that at the apex of the flagellum they always seem to peter out in a way suggestive of damage. The numerical relation of the eleven strands appears to be constant since we have found no other numbers in six comparable specimens.

In comparison with the front flagellum, the hind flagellum of *Saprolegnia* dismembers far less easily. This was also our experience in the biflagellated cells of the brown algae (*Fucus*, Manton and Clarke, 1951*c*; *Pylaiella*, Manton and Clarke, 1951*b*). Fig. 5 is the best of three comparable cases. On the left of the figure, dismemberment is incomplete. The coarse transverse banding visible across the threads is probably the remains of the mechanism by which they are normally attached together (a similar detail will be seen in *Allomyces* below). Dismemberment is, however, fairly complete in the centre of the figure, except for the topmost three strands which, however, can be counted quite clearly where they cross over the others on the right. At this point two of the more internal strands peter out, and therefore the count cannot be fully made at the extreme end of the flagellum. In the centre of the figure it is, however, quite certainly eleven, although whether these include a separate central pair cannot be determined.

This is the first occasion in which we have been able to demonstrate numerical agreement of this kind between the components of two flagella of dissimilar type on one cell, and while there is obviously room for improvement, especially with regard to the hind flagellum, these new observations are a valuable addition to what we were able to achieve in this particular organism without the use of visual staining.

CHLOROSACCUS

By I. Manton, B. Clarke, A. D. Greenwood, *and* E. A. Flint

Chlorosaccus, first described in 1899 by Luther, is a rare genus of yellow-green algae. This group of predominantly freshwater algae, now known as the Xanthophyceae though formerly as the Heterokontae, differs from other algal groups in both structural and physiological characters. In addition, the very characteristic type of motile cell with its two unequal flagella, attached laterally and differing both in length and orientation, recalls the brown algae and water moulds while providing a sharp distinction from the green algae which certain members of the Xanthophyceae otherwise resemble.

It is one of the great achievements of visual microscopy to have demonstrated the presence of a *Flimmergeissel* as the front flagellum in several members of the Xanthophyceae. Vlk (1931 and 1938) showed it in *Heterococcus*, *Botrydiopsis*, *Botrydium*, and *Tribonema*. More recently the spermatozoids of *Vaucheria* have been shown to be of the same type (Koch, 1951), an observation which can scarcely fail to alter the taxonomic treatment of this otherwise very familiar 'green' alga.

No observations have yet been made, so far as we know, on any of these genera with the electron microscope, nor has *Chlorosaccus* itself been previously examined for a *Flimmergeissel* by any method. Its historical importance, as the occasion on which the yellow-green algae were first elevated to the rank of a separate group (cf. Luther, 1899), makes the opportunity for studying it particularly welcome.

The species *Chlorosaccus ulvaceus* Messik. and Vischer, was first found in Switzerland (Messikommer and Vischer, 1946; Messikommer, 1949) and it has recently been detected in England between the months of April and October in two Yorkshire streams (Flint, 1951). When mature, the thallus is an irregular, macroscopic, palmelloid colony, in which the cells are arranged in a single layer within the mucilage. It was noticed that when the alga was brought into the laboratory, many of the cells divide into four, each daughter cell forming a zoospore. The motile cells have two chloroplasts and two flagella, the longer directed forwards and the shorter backwards. A fuller account of the species will be published elsewhere by Flint.

The material for this study was obtained in October, just before the alga disappeared from the streams. The production of zoospores at this time proved to be unpredictable and we did not succeed in making any preparations directly onto electron microscope carriers. Preserved material of zoospores collected earlier in the summer proved unsatisfactory and the results which

follow were all obtained from freshly liberated zoospores killed with osmic vapour and allowed to dry down at once onto clean glass slides, in which condition they were conveyed to Leeds.

Some of these preparations were immediately stained and photographed, thereby providing a demonstration of the *Flimmergeissel* with visual light (cf. Fig. 6, Pl. IV). Others were left unstained to serve as a control, and appropriate pieces of both types of preparation were stripped off as described on p. 206 and remounted for electron microscopy.

Pl. IV shows a photograph (Fig. 6) and electron micrographs at various magnifications (Figs. 7–9) of one and the same stained cell. The cell is smaller than in the previous case of *Saprolegnia* (Fig. 1) and resolution by the light microscope is in consequence less effective. Close comparison of Figs. 6 and 7 will show that the lateral appendages resembling hairs in Fig. 6 are not hairs but shadows corresponding to tufts of hairs. They are therefore not, in this case, a true picture of the *Flimmer*, although they betray its presence.

The detailed effects of staining as seen with the electron microscope are closely comparable to those already seen in *Saprolegnia*. There is an unpleasant deposit in the ground and all parts of the structure have become thickened. The hairs have also in this case become matted together. Their opacity to the electron beam has, however, become notably enhanced.

A truer picture of a good front flagellum in the unstained condition is contained in Fig. 10. The general resemblance to both *Pylaiella* (Manton and Clarke, 1951*b*) and *Saprolegnia* (Manton, Clarke, and Greenwood, 1951) is very striking. The *Flimmer* hairs are mostly borne singly, although the appearance of certain detached hairs in two places in the figure suggests that there are a few pairs. We have not yet been able to determine whether there are hair-points terminating the hairs in *Chlorosaccus* as in the two other cases, but even without this detail the resemblance is close.

Fibrillar disintegration of both flagella was obtained in this material in both the stained and unstained condition. Some examples of both types are assembled on Pls. VI and VII, though here it should be noted that the absolute range of sizes of the micrographs proved to be so great that it has not been practicable to reproduce them all at the same magnification.

A front and a hind flagellum from two different cells are shown unstained in Figs. 11 and 12. The hind flagellum, which is shown complete (Fig. 11), also illustrates the attachment of both flagella to a common basal body visible at the top of the picture and originally inside the cell. The dismembered flagellum, outside the cell, contains eleven strands, including a definite central pair. The front flagellum (Fig. 12) is far too long to be shown complete. It has also dismembered into eleven strands; the *Flimmer* hairs are still in place, but the very undulating course of the central pair of strands recalls that of *Saprolegnia*.

Figs. 13–15 show comparable examples from stained material with the magnifications adjusted for convenient inclusion in the space available. As in other cases of staining, the fine details are less well shown, although the

critical detail of numbers of strands is at least as easy to see. In the front flagellum (Fig. 13) fibrillar disintegration is less perfect than in the unstained specimen and the best places for counting the numbers of strands on the two sides are marked by arrows. The total number is not in doubt: there are again exactly eleven strands, with a very distinct central pair.

Figs. 14 and 15 complete the evidence for *Chlorosaccus* as far as it is necessary to reproduce it (several other specimens of both flagella have been examined without adding anything new). They show two examples of dismembered hind flagella in the stained condition, reproduced at different magnifications to fit the space available. Both show eleven strands, including a central pair, and the only new observations to which attention need be directed are the faint amorphous prolongations visible beyond the apparent tips of the strands in both figures. This appearance has been met with also in one of the fungi to be described below (*Olpidium*) and it is the only indication that we have been able to obtain of material which may perhaps represent the whiplash point of the intact flagellum.

ALLOMYCES AND OLPIDIUM

By A. D. Greenwood *and* B. Clarke

These two fungi may conveniently be taken together since they share a type of zoospore very different from those which we have previously examined, thereby providing as strong a contrast with the preceding as can be obtained from within the Thallophyta. In both *Allomyces* and *Olpidium* there are zoospores with a single, posteriorly directed flagellum: a type of flagellation which is familiar among animal spermatozoids but rare in plants.

Since only the cilia are of importance in the present connexion, the other characters of the fungi in question may be passed over with very brief mention. Both belong to the Phycomycetes, although they are not members of the Saprolegniales. *Allomyces* is a free-living, filamentous, saprophyte, isolatable from soil in several tropical countries (cf. Emerson, 1941) though rare in cooler climates; it is placed in a small group of mainly aquatic fungi, the Blastocladiales. *Olpidium* is a parasite, sometimes on crop plants, and is placed in the family Olpidiaceae of the large and varied group of unicellular fungi, the Chytridiales.

Taking *Allomyces* first, a view of its very large and tadpole-like zoospore is contained in Fig. 16 with certain details of the intact flagellum shown at a higher magnification in Figs. 17 and 18. The material (*A. arbuscula*) had been supplied to us in the form of a stock culture of the sporophytic stage on oatmeal agar by Dr. Webster of Sheffield, to whom we are very grateful for this timely gift.[1] The zoospores hatch out very easily from the so-called thin-walled sporangia when these are placed in water for a few hours, and the preparation was made from live zoospores killed directly on the electron microscope carrier in the usual way. The only significant observations on the structure of

[1] This material originated as a culture from the Centraalbureau voor Schimmelcultur, Baarn.

the intact flagellum to which attention should perhaps be directed are the wide translucent sheath (Fig. 17) and the whiplash tip (Fig. 18). Faint longitudinal striations can just be detected in axis and tip in the latter figure.

Information on fibrillar disintegration of the flagellum in *Allomyces* was obtained from material killed and dried on glass and subsequently stained and transferred as previously described. Figs. 19 and 20 show two examples. Both have the same number of strands, namely eleven, including a central pair. In each case the count must be made near the free end, where dismemberment is most complete. At the other end the attachment to the cell body in both is marked by a characteristic bulbous base which is presumably internal to the body in life. The sheath has vanished and so apparently has the whiplash point, for there is nothing to indicate its presence or nature in the dismembered cells. Certain coarse dark bands, crossing the fibrils in the basal part of the flagellum, again suggest the partial retention of some material binding the fibrils together, as in the case of the hind flagellum of *Saprolegnia* (Fig. 5).

Olpidium has much smaller zoospores, as may be seen from Pl. X. Our material, identified as *O. Brassicae*, was kindly supplied to us as a parasite on infected *Brassica* seedlings by our colleague Mr. Powell Jones of the Agriculture Department of the University. The sporangia can be detected with the low power of the light microscope in the epidermal cells of infected roots and the zoospores hatch out readily on submergence in water. They cannot be obtained easily in such large numbers as in *Allomyces*, but a small cloud of swarmers usually remains for a few moments after discharge, in the water near the exit tube of each dehisced sporangium. With a very finely drawn out pipette zoospore suspensions were collected from such swarms and transferred as small drops either to formvar films on electron microscope carriers or to clean glass slides, where they were killed and dried down in the usual way. In this case it was necessary to wash very thoroughly in distilled water not only the portions of infected root but also the killed and dried preparations on the carriers and the slides to remove dirt and secretions before proceeding with the further operations required for electron microscopy. The direct preparations on the carriers were dried again and shadowed with gold palladium; they yielded the pictures of intact spores in Figs. 21 and 22. Other preparations on glass were stained and by this means, in spite of the small size of the cells, numerous cases of fibrillar disintegration were found in a few of the many drops examined. Favourable specimens were located visually and transferred individually to formvar films on carriers for the electron microscope.

There is little to say about the morphology of the intact spores shown unstained in Figs. 21 and 22 and stained in Figs. 23 and 26. There is no conspicuous sheath to the flagellum, though there is probably a skin which reveals its presence only by a characteristic flexure induced in certain partially decomposed specimens (not shown in the plates), apparently by shrinkage of the skin. The whiplash point is conspicuous (Fig. 23), and in the unstained condition (Fig. 22) the slight terminal swelling on the end of the whiplash is generally encountered and may be characteristic.

Pl. XI shows a few samples of fibrillar disintegration out of about a dozen which have been studied, all in the stained condition. Fig. 26 shows a stained cell from the same batch of material for comparison of sizes. The two low-power views (Figs. 24 and 25) of dismembered cells show very clearly the compound bulbous base which marks the attachment of the flagellum to the inside of the body. The body itself has burst and become spread out into a thin film covering a remarkably large area and taking up the stain very faintly. No interpretable internal organs other than the base of the flagellum have remained.

The dismembered flagella are very elegant objects in this material. The two low-power views (Figs. 24 and 25) were selected for the clarity with which they show general features. More fully dismembered specimens are, however, preferable for the study of numerical details and the tip of one such specimen is shown at a higher magnification in Fig. 27. The full number of eleven strands, including a central pair, is very beautifully displayed and we have several other examples of comparable quality. Wherever dismemberment is complete, in this material as in the others, the same numbers are revealed.

The only other detail to which attention need be directed at the present stage is the appearance, in the stained specimens, as of a smoke-like prolongation beyond the apparent ends of the strands. This is visible in both the low-power views (Figs. 24 and 25) and in the specimen of Fig. 27 at a lower magnification; at high magnifications the substance is so diffuse that it ceases to affect a photographic plate. There is nothing which can be said with certainty about the material of which these prolongations are composed, but as in the case of the stained hind flagella of *Chlorosaccus*, they seem to be genuine and the only suggestion we have to make about them is that they perhaps correspond to the region of the whiplash which, otherwise, cannot be accounted for in the dismembered specimens. If this interpretation is correct, the whiplash would be composed of the same eleven strands as the rest of the flagellum but in a much more destructible condition.

DISCUSSION

Several of the details recorded above could profitably be investigated further and they need this before serious discussion is attempted. Examples of topics in this condition are the structure of the whiplash, the structure and function of the bulbous bases, and the nature of the coarse cross-banding seen after dismemberment in *Allomyces* and the hind flagellum of *Saprolegnia*.

In other cases the details are of technical rather than of descriptive interest. The observations on staining are of this kind. It is perhaps, however, of some general scientific interest to have revealed one at least of the reasons for the long controversy over the reality of the *Flimmer* (for quotation of literature, see Manton and Clarke, 1950). Had all cells possessing it been as large as the zoospores of *Saprolegnia*, general acceptance would probably have come sooner. Even here, however, as has been shown, the observation lies so near to the limit of resolution with the light microscope that on a smaller cell (as in

Chlorosaccus) a definitely false image is obtained with this instrument which, quite rightly, can be criticized as untrustworthy, as in details it is. Another cause of the earlier doubt is probably the ease with which the hairs can be destroyed. This was the reason why we were unable to work with material of *Chlorosaccus* preserved in formalin either with or without previous killing with osmic vapour. Even in the dried material in which most cells showed some traces of hairs, there was a considerable range of quality of condition—a circumstance which is by no means unusual in *Saprolegnia* and the brown algae. Destruction of the *Flimmer* by unsuccessful handling therefore is certainly one reason for possible failure to demonstrate it. Negative evidence is therefore less trustworthy than positive evidence with the light microscope, unless in the hands of an experienced investigator.

Over and above these details, however, two major conclusions stand out from the facts presented which deserve some further comment. On the one hand we have the surprising observation that in each of four organisms the flagella, whether directed forwards or backwards, and whether of the *Flimmergeissel* type and provided with hairs, or of the *Peitschgeissel* type and provided with a whiplash point, decompose with monotonous uniformity into eleven strands. The meaning of this is as inscrutable as it has been on previous occasions. The examples of it are, however, now so numerous and varied that it is no longer a fact to be noticed in passing as in Manton and Clarke (1951*b*), but a major generalization which cannot be ignored. In our own previous papers we have found it in ferns (Manton and Clarke, 1951*a*), *Pylaiella* (Manton and Clarke, 1951*b*), *Fucus* (Manton and Clarke, 1951*c*), and *Ulothrix* (Manton, 1951), representing land plants, brown algae, and green algae. We have now had the same result for three fungi and a yellow-green alga, and the only discordant record in this whole array is in the hind flagellum of *Fucus* for which we obtained only a single example in Manton and Clarke (1951*c*), and which seemed to show somewhat fewer strands. In the light of the present position it is clear that the exceptions are now the cases which need more intensive study to confirm them or explain them.

What has been said for plants can be repeated in animals. We have nothing at present to add to the summary of animal results collected from the literature quoted in Manton and Clarke (1951*c*). It is, therefore sufficient to say that eleven strands have been recorded several times in animals, though nine and twelve have also been described. Without at the moment wishing to comment on the exceptions, the overwhelming prevalence of eleven, in the form of nine strands and a central pair, is such that one is forced to conclude that there must be a fundamental functional significance in this number and that it cannot be merely a resemblance due to phyletic affinity.

With regard to the other observations, on hairy flagella, the conclusion is otherwise. If comparison is made with our own pictures for *Pylaiella* (Manton and Clarke, 1951*b*), *Ochromonas* (Manton, 1951), *Saprolegnia* (Manton, Clarke, and Greenwood, 1951), and now *Chlorosaccus*, the resemblance between the front flagella in all these is very striking and it extends to quite elaborate

details. Since this type of flagellum is unknown in animals and does not occur in land plants, green algae, or indeed any other group of multicellular plants at present known, the conclusion seems inescapable that the brown algae, yellow-green algae, and certain groups of fungi and flagellates are in fact phyletically related more directly with each other than they are to other groups.

This conclusion has already been drawn, in whole or in part, by those light microscopists who have most effectively studied these structures, notably by Vlk (1931, 1938, 1939) and Couch (1941), with their collaborators (notably Longest, 1946, and Koch, 1951), though few writers of textbooks (Bessey, 1950, is a notable exception) have taken the evidence seriously. Our own independent evidence obtained from the electron microscope study of brown algae, *Saprolegnia* among fungi and the Chrysophycean *Ochromonas* among flagellates, was summarized in a preliminary way in July 1951 and shown to point in the same direction (Manton, 1951). The addition now of the Xanthophyceae as represented by *Chlorosaccus* to the electron microscope evidence strengthens it considerably. So also does the demonstration with the light microscope of a comparable structure in *Vaucheria* by Koch (1951), to which attention was directed on p. 208. The sum of both types of evidence is now, in our view, so strong as to be incontrovertible and we put this conclusion forward as the most important botanical, as opposed to biophysical, result of our present series of investigations.

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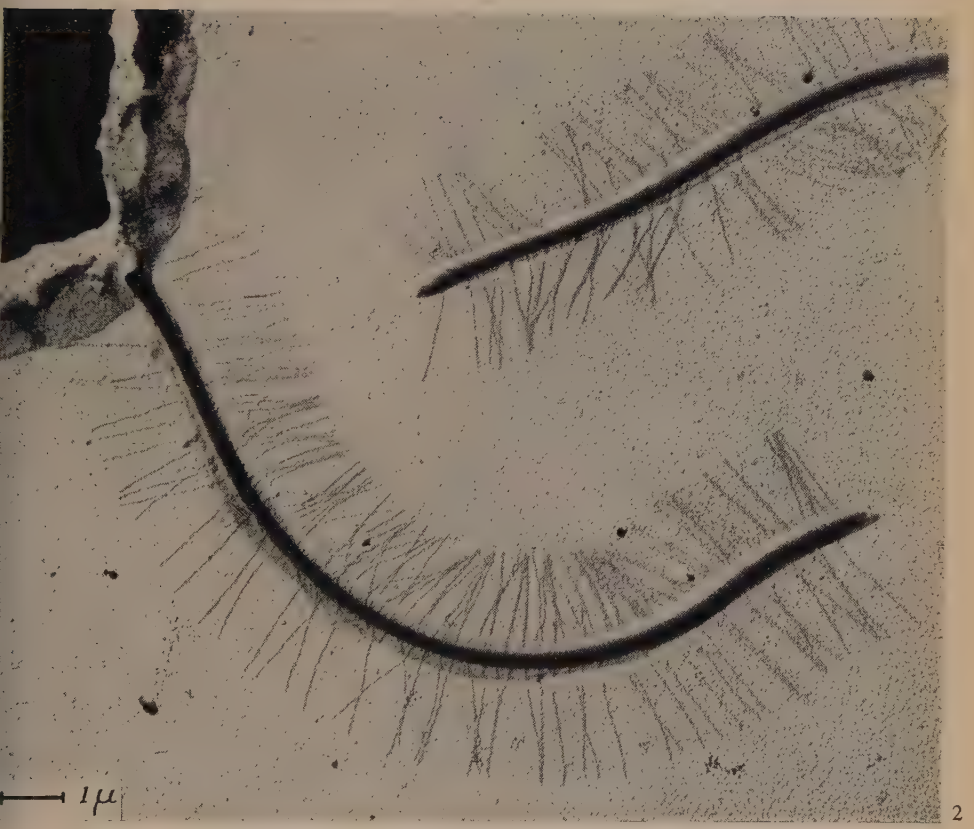

2

I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 1. First-stage zoospores of *Saprolegnia ferax* photographed with visual light under an oil-immersion objective after bacteriological staining. The two dissimilar flagella clearly displayed. Magnification ×2,000.

FIG. 2. The same specimen as Fig. 1 examined with the electron microscope. Exposure M67.14, 60 kV., magnification ×8,000.

PLATE I

3

I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 3. More highly magnified portion of the left-hand front flagellum of the specimen of Figs. 1 and 2, to show in detail the effect of the mordant and staining. An exactly comparable unstained specimen is available for comparison in Fig. 6 of the previous paper. Electron micrograph M67.17, 60 kV., magnification ×20,000.

PLATE II

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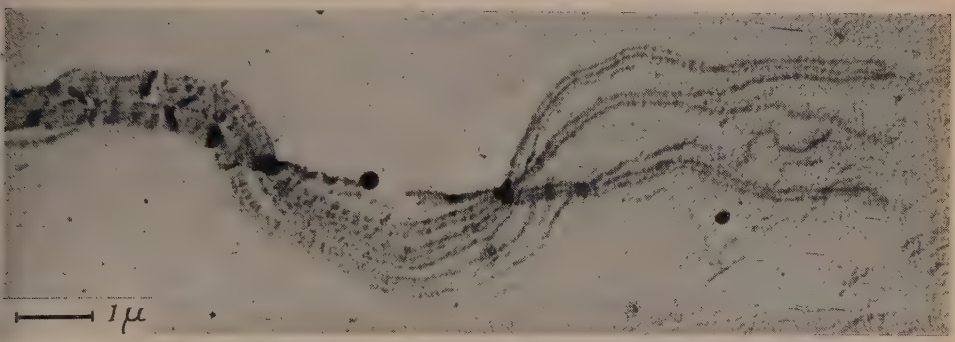

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I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 4. *Saprolegnia ferax*, a disintegrated front flagellum of the first stage, stripped from glass after staining. Electron micrograph M75.1, 60 kV., magnification × 10,000.

FIG. 5. Distal end of the hind flagellum from the same cell as Fig. 4. Electron micrograph M73.8, 60 kV., magnification × 10,000.

PLATE III

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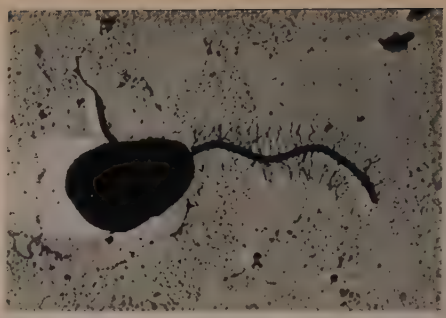

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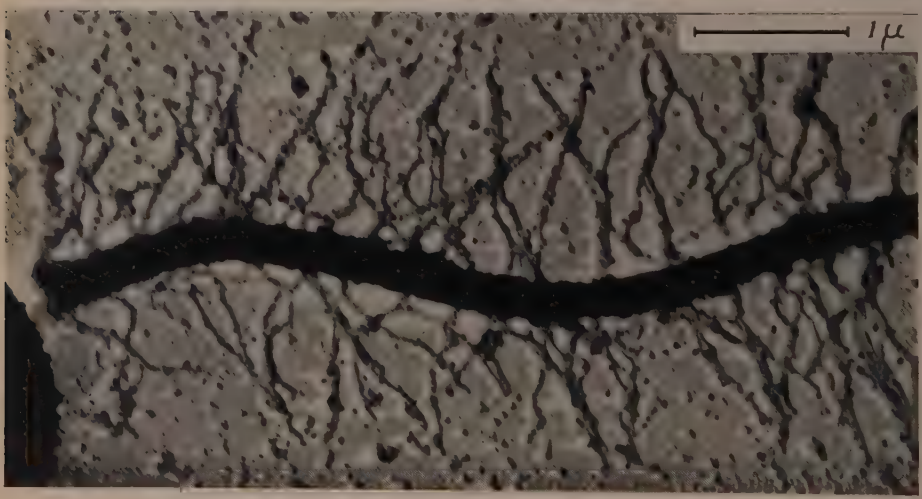

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FIG. 6. *Chlorosaccus ulvaceus*, a stained zoospore, photographed under oil immersion with the light microscope. Magnification × 2,000.

FIG. 7. The same cell as Fig. 6 under a low-power of the electron microscope. Electron micrograph M66.18, 60 kV., magnification × 3,000.

FIG. 8. The front flagellum of the same cell more highly magnified. Electron micrograph M66.22, 60 kV., magnification × 10,000.

FIG. 9. The same, still more highly magnified. Electron micrograph M66.24, 60 kV., magnification 20,000.

PLATE IV

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I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 10. *Chlorosaccus ulvaceus*, the front end of an unstained front flagellum. Reversed print of electron micrographs M68.30 and 31, 40 kV., magnification ×20,000.

PLATE V

11

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I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 11. *Chlorosaccus ulvaceus*, an unstained hind flagellum showing fibrillar disintegration and part of the intracellular system attaching the two flagella to each other and to the body. Reversed print of electron micrograph M72.11, 60 kV., magnification × 12,000.

FIG. 12. Part of an unstained front flagellum from another cell on the same preparation as the preceding. Reversed print of electron micrograph M72.17, 60 kV., magnification × 12,500.

PLATE VI

14

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I. MANTON, B. CLARKE, AND A. D. GREENWOOD

FIG. 13. *Chlorosaccus ulvaceus*. Part of a stained front flagellum showing fibrillar disintegration; the arrows show the regions where counts of fibrils on the two sides can best be made. Electron micrograph M75.5, 60 kV., magnification × 7,500.

FIG. 14. Free portion of the hind flagellum belonging to the same cell as the preceding. Electron micrograph M74.24, 40 kV., magnification × 10,000.

FIG. 15. Another specimen comparable to the preceding but at a lower magnification. Electron micrograph M74.21, 40 kV., magnification × 5,000.

PLATE VII

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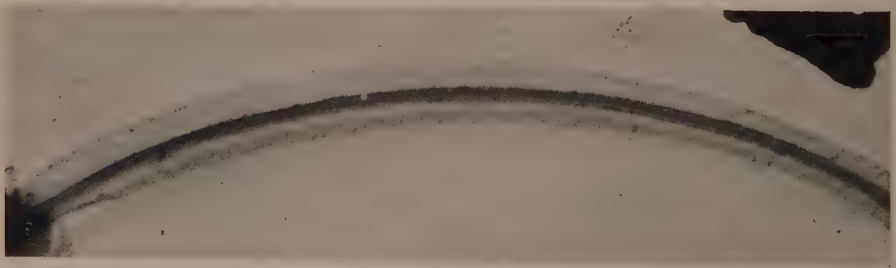

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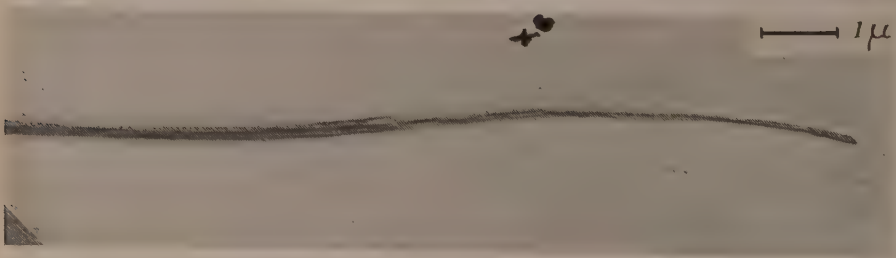

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FIG. 16. *Allomyces arbuscula*. General view of a zoospore and a fine piece of the non-septate vegetative mycelium, unstained. Electron micrograph M59.10, 60 kV., magnification × 3,000.
FIG. 17. More enlarged view of the base of the flagellum of the preceding. Electron micrograph M59.12, 60 kV., magnification × 10,000.
FIG. 18. The same, the tip of the flagellum. Electron micrograph M59.14, 60 kV., magnification × 10,000.

PLATE VIII

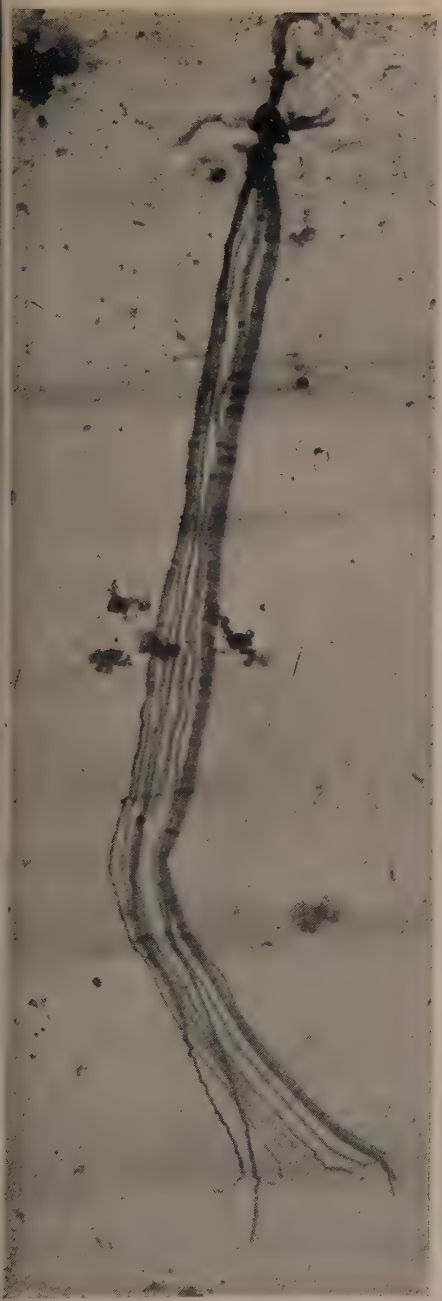

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FIG. 19. *Allomyces arbuscula*, a dismembered flagellum, stained. Electron micrograph M63.4, 60 kV., magnification ×6,000.

FIG. 20. Another specimen of the same. Electron micrograph M63.1, 60 kV., magnification ×6,000.

PLATE IX

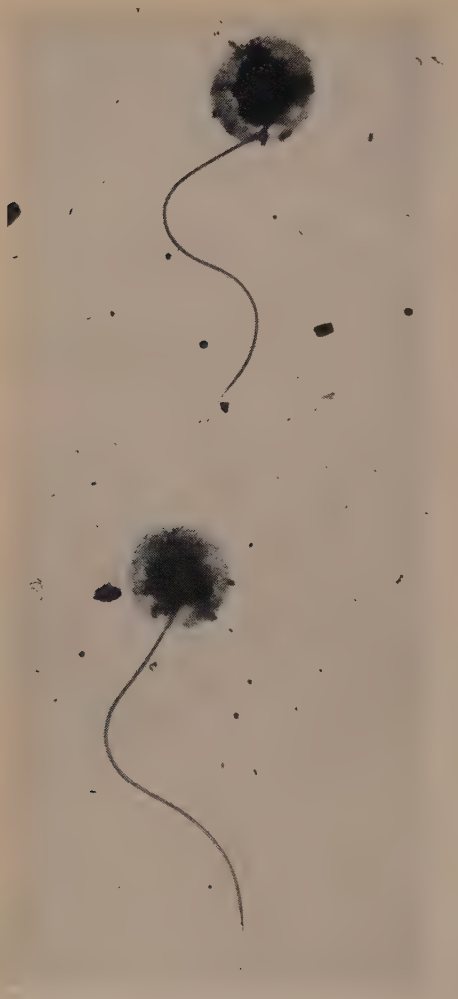

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FIG. 21. *Olpidium Brassicae*, general view of two zoospores, unstained. Electron micrograph M73.28, 60 kV., magnification × 3,000.

FIG. 22. More enlarged view of the tip of the flagellum from the lower cell in the preceding. Electron micrograph M73.30, 60 kV., magnification × 10,000.

FIG. 23. Tip of a flagellum from comparable material, stained. Electron micrograph M70.7, 60 kV., magnification × 10,000.

PLATE X

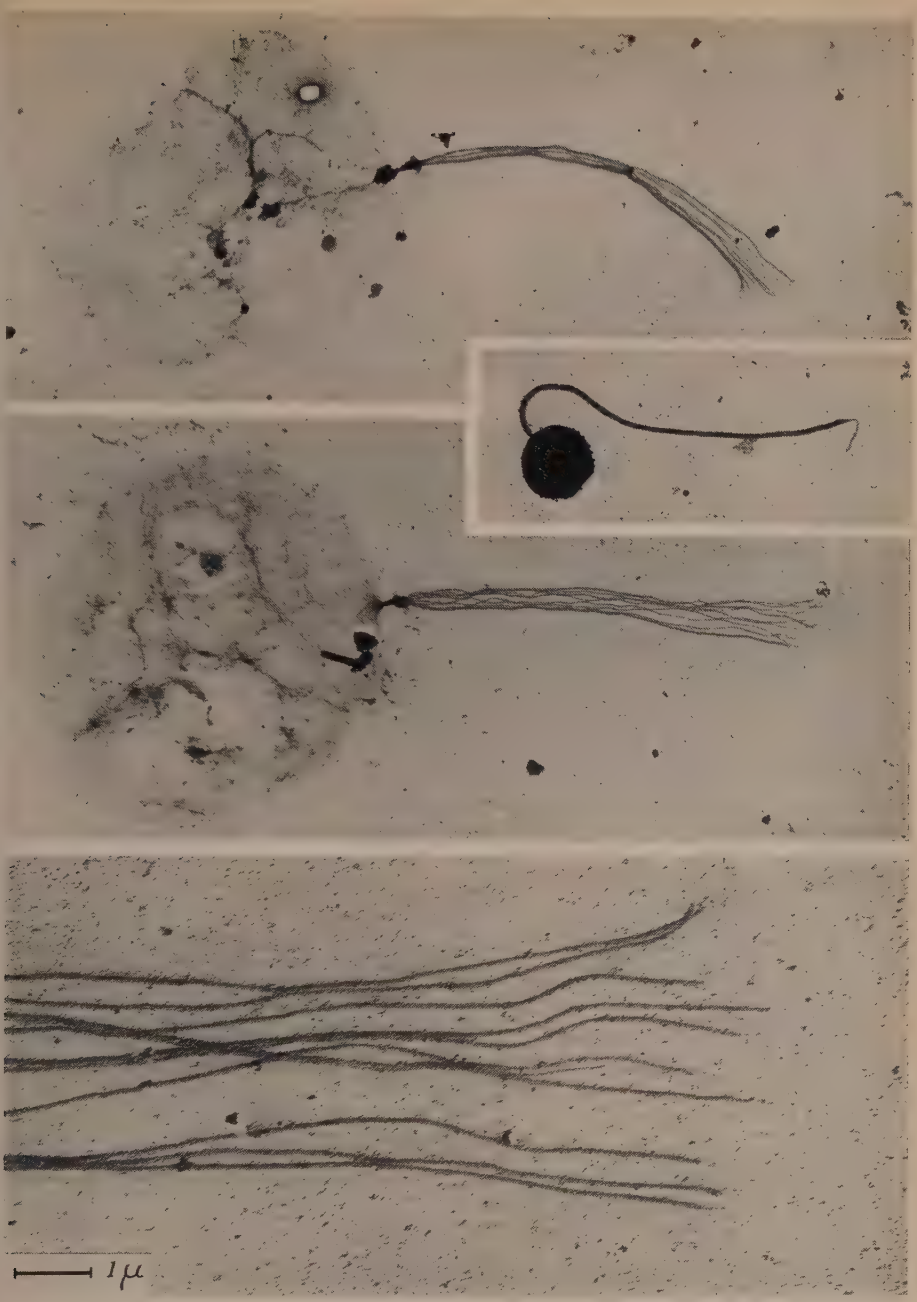

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FIG. 24. *Olpidium Brassicae*. Low-power view of a burst cell with a disintegrated flagellum still attached, stained. Electron micrograph M70.25, 60 kV., magnification ×3,000.

FIG. 25. Another comparable specimen. Electron micrograph M70.21, 60 kV., magnification ×3,000.

FIG. 26. An intact cell from the same stained slide as the preceding. Electron micrograph M70.1, 60 kV., magnification ×3,000.

FIG. 27. Part of a fully dismembered flagellum otherwise comparable to the above, showing a very perfect count of the number of strands. Electron micrograph M72.4, 60 kV., magnification ×10,000.

PLATE XI

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Metabolism of Slices of the Tomato Stem[1]

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SUMMARY

The rate of respiration of tomato stem slices varied considerably, the highest values (Q_{O_2}, 2–3) being obtained for plants in a good nutritional state, and the lowest (Q_{O_2}, 1) in starved plants. The respiratory quotient of 1·0 remained constant.

Glucose fermentation was found to follow both glycolytic and alcohol fermentation pathways, the ratio of ethyl alcohol : lactic acid being 6·6 : 1. Fermentation seems to take place according to the Embden–Meyerhof scheme, as shown by the presence of some of these enzymes operative in this scheme and by inhibition experiments. In the presence of oxygen there was no formation of alcohol or lactic acid.

Pyruvate added to tomato stem slices was metabolized by direct oxidation to acetic acid and by dismutation to lactic and acetic acids and CO_2. The metabolism of acetic acid was demonstrated by its condensation with oxaloacetic acid to form citrate, this being the second time that synthesis of citric acid by this mechanism has been found in plants. The presence of aconitase, of *iso*citric dehydrogenase, of succinic dehydrogenase, and of malic dehydrogenase, as well as the inhibition of respiration by malonic acid, favour the hypothesis that oxidation of carbohydrate in tomato stem slices proceeds via the citric acid cycle. The possibility of an auxiliary route, the malic acid oxidation pathway, also was demonstrated. Tomato stem tissue anaerobically split malic acid into glycolic acid. The further oxidation of glycolic, glyoxylic, and formic acid was demonstrated.

In experiments with C^{14}-labelled acetate and butyrate a dilution of the C^{14}-labelled acids was found after incubation indicating new formation of these acids and of active participation of fatty acid metabolism in the metabolic activities of the tissue.

With the exception of alanine, added amino acids produced a definite increase in O_2 uptake without extra formation of ammonia.

Experimental demonstration of the possibility of electron transport from substrate to molecular oxygen in respiration via polyphenol oxidase was provided by the attainment in a tomato tissue homogenate of a coupled oxidation-reduction between α-ketoglutarate and catechol with DPN and tyrosinase as the catalysts. The presence of cytochrome oxidase was also demonstrated. Thus both systems possibly may take part in the respiration of tomato stem slices.

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INTRODUCTION

THIS paper presents the results of studies of carbohydrate, fat, and amino-acid metabolism of the tomato stem (*Lycopersicon esculentum* Mill. var. 'Bonny Best'). It also integrates the results of isolated investigations of the metabolic reactions in various organs of the tomato plant in an attempt to indicate possible pathways of metabolism in the tissues of the stem. The pertinent literature is referred to in the experimental section and the discussion essential to subsequent studies of the metabolism of tomato crown gall.

EXPERIMENTAL

General

All tomato plants used in this study were grown from certified seed of the 'Bonny Best' variety. The seed was sown in flats of loam soil in the greenhouses of the University of Chicago during all seasons and the plants used when they were in the 3–6 leaf stage (3–5 weeks after germination depending on the season). Only the hypocotyls and first internodes were taken for this work (these are designated as tomato stems) since they tended to be relatively uniform in development and are the first axis segments to mature (Link and Eggers, 1941). For most experiments these organs were cut into thin (0·5–1·0 mm.) disks normal to the long axis of the plant. Slices were collected in water or buffer at room temperature, the water quickly removed through a porcelain filter, and the disks used within 30 minutes after slicing. Procedures for the preparation of homogenates, extracts, &c., are in the text.

Extensive preliminary study indicated that a buffer consisting of Ringer solution at half-normal strength containing 0·01 M. PO_4 at pH 5·3 for oxygen uptake studies and pH 6·0 for fermentation gives optimal gas exchange values with no evident tissue damage. On either side of these values there were marked decreases in the rates of gas exchange. Unless otherwise noted, experiments were performed at 28° C. with air or oxygen-free nitrogen as gas phases. Concentration of all reactants will be given as final concentration except where stated otherwise. Chemical determinations were carried out by methods detailed by Barron *et al.* (1950). Diphosphopyridine nucleotide (DPN) (60 per cent. purity) was obtained from Schwarz & Co., New York; triphosphopyridine nucleotide (TPN) (45 per cent. purity) and *iso*citric acid were kindly given to one of us by Dr. S. Ochoa; cytochrome *c* was obtained from Sigma Chemical Co.; α-ketoglutaric acid and *cis*-aconitic acid were prepared in the laboratory, as were C^{14}-labelled acetate and butyrate which were made from $BaC^{14}O_3$. The determination of glycolic acid was made by an application of the colour test given by Feigl (1937), namely, the formation of a violet colour when 2:7-dihydroxynaphthalene in concentrated H_2SO_4 is heated with glycolic acid. The plant tissue was treated with an equal volume of 10 per cent. CCl_3COOH. After filtration, 0·1 ml. of the filtrate was transferred to a test-tube; to it were added 0·9 ml. of water and 4 ml. of the reagent (10 mg. of 2:7 dihydroxynaphthalene dissolved in 100 ml. concentrated H_2SO_4 immediately before use). The reagent was added dropwise to the test-tube

which was kept in an ice bath and shaken steadily. It was then heated for 15 minutes in a boiling water bath. At the end of this time it was cooled and the colour intensity was measured with a Beckman spectrophotometer at 530 mμ. The colour faded slowly and about 5 per cent. was lost at the end of 1 hour. Malate, pyruvate, formate, oxalate, citrate, or succinate, all at 0·005 M. had no effect on the colour reaction. Acetaldehyde gave a bluish colour which did not absorb light appreciably at 530 mμ. Tissue dry weights were determined after 18 hours at 110° C.

Respiration of tomato stem slices

In marked contrast to the reproducibility of the values for respiration of potato tuber slices (Barron *et al.*, 1950), considerable day-to-day variation was found in tomato-stem slices. The state of nutrition of the plants probably was the main factor in this difference. The endogenous respiratory quotient, however, remained more or less constant, close to one, at pH values from 5 to 6. At a pH value above 7, there was a sharp reduction (Table I).

TABLE I

Effect of pH on the respiratory quotient of tomato stem slices (experiments performed in the same harvest of plants)

Buffer, half-strength Ringer solution+0·01 M. phosphate

pH	Q_{O_2}	Q_{CO_2}	R.Q.
5·3	2·50	2·38	0·95
5·3	2·93	3·10	1·06
6·0	1·87	1·85	0·99
7·3	1·60	1·00	0·62

Anaerobic phase of carbohydrate metabolism

The anaerobic fermentation of tomato stem slices ended, like that of potato tuber slices, with the formation of ethyl alcohol and lactic acid. In these experiments the slices were incubated in Warburg vessels in Ringer-bicarbonate with N_2:CO_2(95:5) as the gas phase. At the conclusion of the incubation period lactic acid and ethyl alcohol determinations were made on the fluid. Ethyl alcohol accounted for 75 per cent. of the CO_2 produced (Gay Lussac equation: $C_6H_{12}O_6 \rightarrow 2CH_3CH_2OH + 2CO_2$). Lactic acid formation corresponded to 13·7 per cent.

$$(CH_3CHOHCOOH + NaHCO_3 = CH_3CHOHCOONa + H_2CO_3)$$

The origin of the residual CO_2, which was 11·3 per cent., was not identified (Fig. 1). The ethanol:lactic acid ratio was 6·6:1, lower than that found in potato tuber slices, which was 17:1. When the experiments were performed in the presence of oxygen neither alcohol nor lactic acid was detected.

Attempts to isolate tomato phosphorylase by techniques used for other plant tissues were unsuccessful. Histo-chemical localization of the sites of phosphorylase activity was accomplished by use of the slice incubation technique of Yin and Sun (1948) after depleting the starch content of the plants by holding them for 5 days in a dark room. Starch granules were formed from

glucose-1-phosphate in all parenchymatous tissues. Foster and Tatum (1938) found that starch did not usually accumulate to any extent in tomato stem tissues unless the plants were held at low temperatures. Evidently sucrose, not starch, is the primary carbohydrate reserve in tomato stems (Went, 1946). Invertase, which hydrolyses sucrose to glucose and fructose, has been observed in tomato stem and leaf tissues for many years.

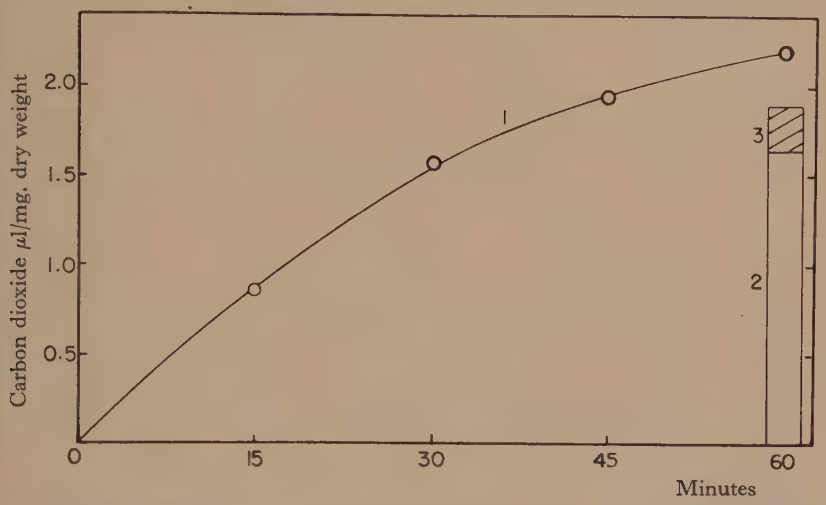

FIG. 1. Anaerobic fermentation of tomato stem slices. Buffer, Ringer–bicarbonate, pH 6·0. Gas phase, N_2:CO_2(95:5). Temp. 28° C. 1. CO_2 production. 2. Alcohol formation. 3. Lactic acid formation

Phosphorylation of glucose and fructose was followed manometrically in tomato tissues. Stems were homogenized in a Waring Blendor and the suspension was passed through cheesecloth to remove fibres. One-millilitre portions of this suspension were added to manometer vessels containing $NaHCO_3$, 0·02 M.; acetate, 0·05 M.; NaF, 0·01 M.; Mg^{++}, 0·0001 M.; ATP, 0·003 M., and either glucose or fructose, 0·01 M., or water. The vessel contents were saturated with O_2-free N_2:CO_2(95:5), and CO_2 formation was followed for 30 minutes (Fig. 2). This CO_2 represents the formation of glucose or fructose phosphoric acid. Hexoses or ATP alone did not increase the rate of CO_2 formation. Additional indirect evidence for the presence of hexokinase was obtained by the addition of 0·01 M. *dl*-glyceraldehyde (Rudney, 1949) to fermenting slices. In Teorell-Stenhagen buffer (1938), pH 4·0, a 25 per cent. inhibition of fermentative CO_2 formation was observed in 2 hours. Sisakyan and Kabyakova (1949) have reported the presence of phosphoglucomutase in tomato chloroplasts. Aldolase, which splits hexose-diphosphate to aldo- and keto-trioses, was found by Tewfik and Stumpf (1949) in the seeds, leaves, and fruit of the tomato plant. Using 0·003 M. iodoacetate, Caldwell and Meiklejohn (1937*b*) obtained a 77 per cent. inhibition of oxygen uptake by tomato stems. When iodoacetate or lewisite, both thiol reagents, were tested for their effects on stem slices anaerobically, a 37–40 per cent. inhibition of CO_2

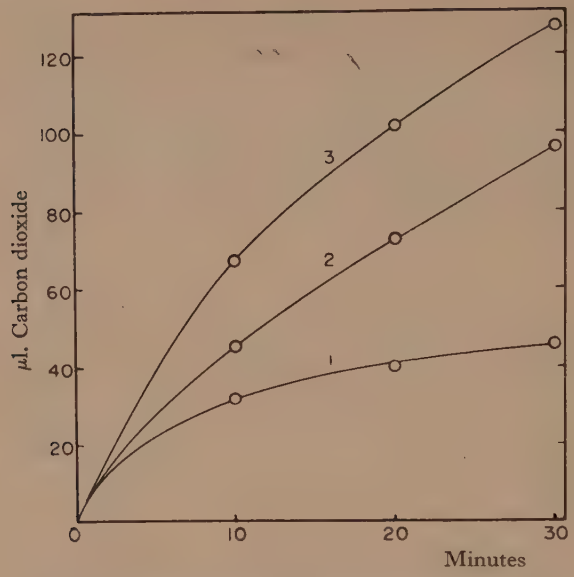

FIG. 2. Phosphorylation of glucose and fructose by tomato stem tissues. 1. Control (absence of hexoses). 2. Glucose. 3. Fructose

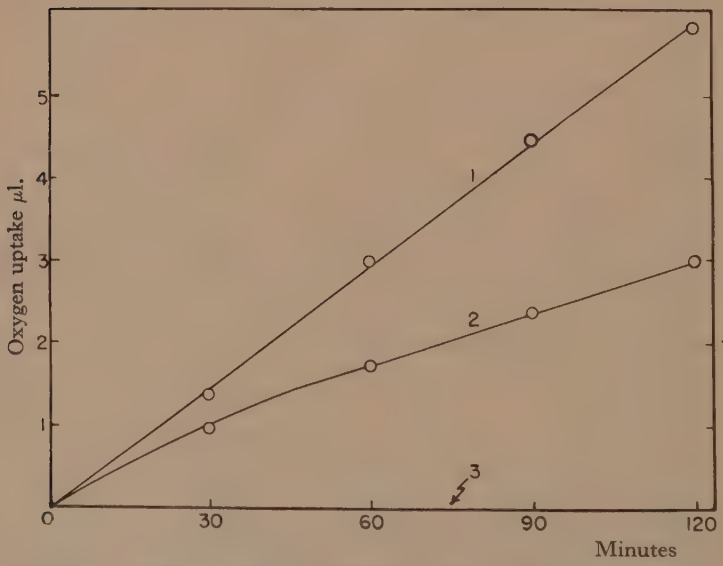

FIG. 3. Effect of NaF on oxygen uptake of tomato stem slices. Inhibitor prepared in buffer at pH 5·4. 1. Control. 2. NaF, 0·005 M. 3. NaF, 0·01 M.

formation with 0·001 M. inhibitor was obtained; since phosphoglyceraldehyde dehydrogenase is an -SH enzyme, these experiments may be taken as an indication of inhibition of this enzyme by the -SH reagents.

Fig. 3 shows the effect of NaF on the oxygen uptake of tomato stem tissues.

Caldwell and Meiklejohn also obtained extensive inhibition with this compound. Such inhibition has usually been interpreted as due to inhibition of enolase (Warburg and Christian, 1942).

The main oxidative pathway of carbohydrate metabolism

a. Pyruvate. The multi-modal reactivity of pyruvic acid was pointed out by Barron (1940). In oxidation-reduction reactions it may be oxidized to acetic acid; it may undergo dismutation to lactic and acetic acid; it may be reduced to lactic acid; or it may be carboxylatively reduced to malic acid. It may also be decarboxylated to acetaldehyde. The relative amounts of oxidation and dismutation may be measured by determining the oxi-dismutation coefficient (Pyruvate utilized in O_2/Pyruvate utilized in N_2) (Barron and Lyman, 1939). Pyruvate utilization was greater in the presence of O_2 than in its absence, as seen by the oxi-dismutation coefficient of 1·51 (Expt. 1, Table II). In the absence of O_2, the reaction was mainly one of dismutation, as shown by lactic acid and CO_2 formation ($2CH_3COCOOH \rightarrow CH_3CHOHCOOH + CH_3COOH + CO_2$). Very little acetaldehyde formation was observed in spite of the presence of carboxylase in the tissue (Expt. 2, Table II). Similar results were obtained by Barron, Ardao, and Hearon (1950) on addition of pyruvate to washed baker's yeast. Malonate 0·05 M. inhibited pyruvate oxidation 87 per cent. in experiments performed at pH 5·3.

Evidence for the oxidative decarboxylation of pyruvate ($CH_3COCOOH + 1/2\ O_2 \rightarrow CH_3COOH + CO_2$) was obtained by the determination of acetic acid in the presence and absence of fluoroacetate. Bartlett and Barron (1947) found that the addition of fluoroacetate to tissue slices incubated with pyruvate produced an accumulation of acetate. On incubation of tomato stem slices with pyruvate, there was definite formation of acetate even in absence of fluoroacetate; in its presence acetate formation was increased (Expt. 3, Table II).

The relatively low metabolism of pyruvate probably is due to lack of penetration of the dissociated ion CH_3COCOO^-. In fact, on incubation of

TABLE II

Utilization of pyruvate by tomato stem slices

In aerobiosis, Ringer-phosphate, pH 5·3 with air as gas phase. In anaerobiosis, Ringer-phosphate with N_2 as gas phase. Pyruvate, 0·001 M. Temp. 28° C. Values give μl. per mg. dry weight per hour.

Substance	Utilization or formation	
	Aerobiosis μl.	Anaerobiosis μl.
1. Pyruvate utilization	2·12	1·40
2. Pyruvate utilization	—	0·38
Lactate formation	—	0·148
Acetaldehyde formation	—	0·011
3. Pyruvate utilization	0·43	
Same in 0·01 M. fluoroacetate	0·37	
Acetate formation	0·075	
Same in fluoroacetate	0·105	

tomato stem slices with pyruvate at different pH values it was found that its utilization increased as the pH approached the pK value of pyruvic acid (Fig. 4). As in yeast cells (Barron, Ardao, and Hearon, 1950) pyruvate seems to penetrate the tomato cell membrane only as the undissociated acid.

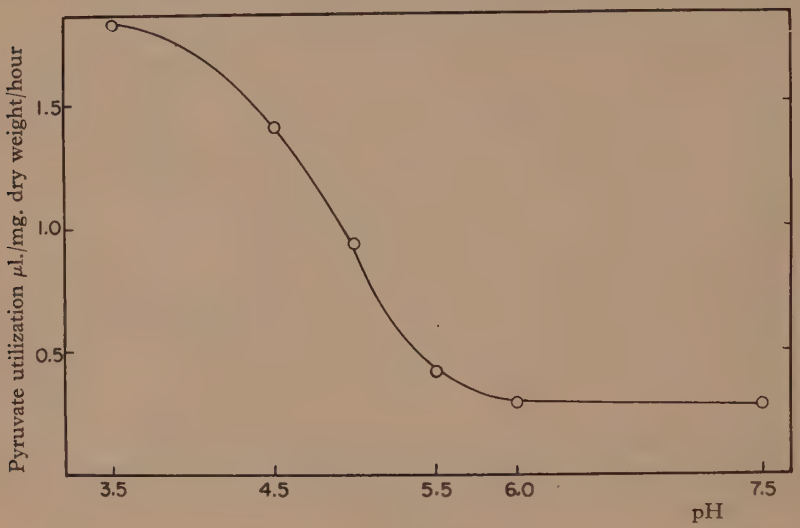

FIG. 4. Effect of pH on the aerobic utilization of pyruvic acid. Buffer, Teorell–Stenhagen. Pyruvate, 0·001 M. in buffer. Temp. 28° C. Reaction time, 2 hours

b. Acetate. It has been shown that on addition of pyruvate to tomato stem slices there is some accumulation of acetate. When acetate was added to tomato stem slices at low pH values (3·5 and 4·0) there was an increase in the oxygen uptake and a vigorous acetate utilization. Moreover, addition of malonate definitely inhibited this oxidation (Table III). Further evidence of the oxidation of acetate was obtained with the aid of C^{14}-labelled acetate, $CH_3C^{14}OONa$. On incubation at pH 5·3, although there was a decrease of O_2 uptake, acetate was utilized and the respiratory CO_2 contained $C^{14}O_2$ (Table IV). An unexpected finding was the considerable dilution of the radioactive acetate at the end of the experiment, an indication of new formation of acetate by the tissue. This dilution is another indication that the volatile acid formed on addition of pyruvate probably was acetate.

TABLE III

Acetate metabolism in tomato stem slices and the effect of malonate

Buffer, Teorell–Stenhagen, pH 3·5. Figures give O_2 uptake or Acetate utilization in μl./mg. dry weight per hour.

O_2 uptake, no substrate	2·33
O_2 uptake + malonate	1·74
O_2 uptake + acetate	2·35
O_2 uptake + acetate + malonate	1·63
Acetate utilization	6·5

TABLE IV

Utilization of $CH_3C^{14}OONa$ *by tomato stem slices*

Buffer, Ringer-phosphate, pH 5·3, 4·5 ml. Acetate 0·5 ml. containing 50 μM. Tissue slices, 61·2 mg. dry weight. Temp. 28°. Incubation time, 2 hours

Experimental	Acetate utilization or O_2 uptake, μl.	C^{14} counts/minute
Tissue, no acetate, O_2 uptake	356	—
Tissue with acetate O_2 uptake	292	—
Acetate utilization	300	—
C^{14} acetate initial	—	404, 960
C^{14} acetate final	—	296, 350
$C^{14}O_2$, respiratory	—	520

c. Citrate. The oxidation of acetate starts with its condensation with oxalacetate to give citrate (Stern and Ochoa, 1949). Experiments were performed according to the conditions chosen by Barron *et al.* (1950) when they demonstrated the synthesis of citric acid from acetate and oxalacetate in potato tuber tissues. Twelve grammes of tomato stem were homogenized in the presence of 50 ml. of Ringer-phosphate, pH 5·3. The suspension was passed through cheesecloth. To 6 ml. of this suspension were added 0·005 M. acetate and oxalacetate, 0·05 M. malonate, and 0·001 M. adenosine triphosphate. The vessels were incubated at 30°, and samples were withdrawn for citric acid analysis 30 and 60 minutes after incubation. Citric acid formation increased with the time of incubation (Fig. 5). To date this is the second demonstration in plants of acetate oxidation through citric acid formation, the first having been found in tissues of the potato tuber, another member of the Solanaceae. Kaplan and Lipmann (1948) have demonstrated the presence of coenzyme A in tomato fruit tissues.

There is some evidence that citric acid forms a stable substrate pool in tomato stem tissues. Pierce and Appleman (1943) found 12·3 milli-equivalents of citric acid per 100 g. of dry weight of tissue. Although the addition of

TABLE V

Utilization of intermediates of the citric acid cycle

Figures give μl./mg. dry weight of tomato stem slices per hour, except those marked with an asterisk which were obtained with the juice of the ground stem.

Substrate	pH	O_2 uptake or CO_2 formation		Utilization
		No substrate μl.	Substrate μl.	
Citrate	4·5	2·7	2·8	5·96
α-Ketoglutarate	5·3	2·5	2·5	4·5
Succinate,* O_2 uptake	7·4	3·0	29·5	—
Malate	5·4	2·6	3·25	—
Malate,* CO_2 formation	7·4	75	124·5	24·7
Oxalacetate,* CO_2 formation in 20 minutes	5·0	98	125	—

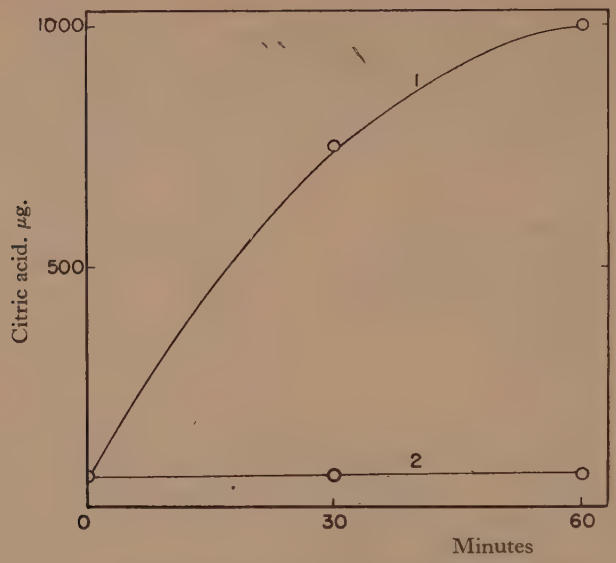

FIG. 5. Formation of citrate by tomato stem homogenate. 1. Complete system. 2. Incomplete systems

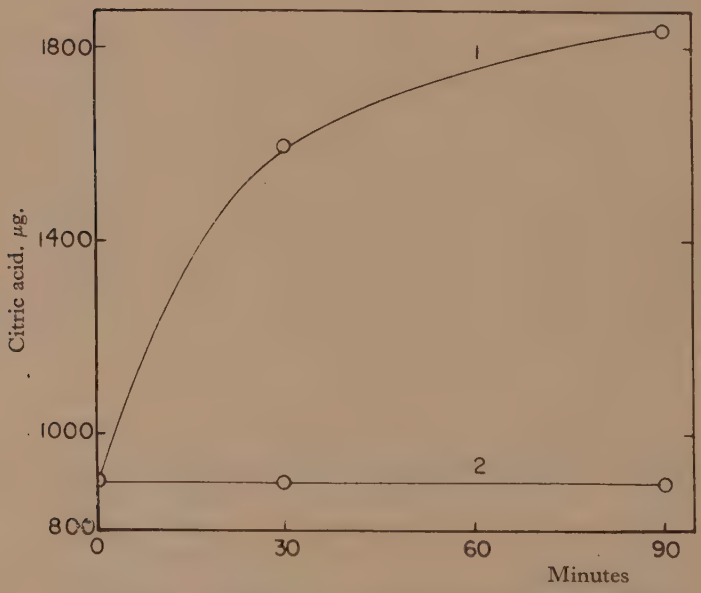

FIG. 6. Activity of aconitase of tomato stem tissues. 1. Tissue extract in the presence of *cis*-aconitate. 2. Same without *cis*-aconitate

citrate (0·001 M., pH 4·5) did not increase the oxygen uptake, there was utilization of this substrate (Table V). The oxidation of citrate probably occurs as in all cells by conversion to *iso*citrate. In fact, the presence of aconitase, the enzyme which catalyses the establishment of an equilibrium between citric

cis-aconitic, and *d-iso*citric acids, was demonstrated in tomato stem tissues. The enzyme was extracted essentially according to Johnson (1939). On incubation with *cis*-aconitic acid there was steady formation of citric acid (Fig. 6).

It is known that a large number of seeds contain *iso*citric dehydrogenase (Wagner-Jauregg and Rauen, 1935). This enzyme is also present in the tomato stem tissue. Thirty grammes of tomato stem tissue were ground with sand at 3° C., and the fluid thus obtained was passed through cheesecloth. To the central portion of Thunberg tubes were added 1 ml. of the filtered tissue suspension; 3·0 ml. of 0·01 M. phosphate, pH 7; 0·3 ml. of Na *iso*citrate, containing 4·16 μmols.; 0·3 ml. of Na adenosine triphosphate, containing 3·47 μmols.; and 1 ml. of 0·0001 M. methylene blue. The side arm of the Thunberg tubes contained 0·4 ml. of a neutral solution of 450 μg. of TPN. The control tubes contained no *iso*citric acid. After evacuation the TPN solution was mixed with the contents of the central tube and the tubes were incubated at 38° C. The dye was reduced in 19 minutes in the presence of *iso*citrate, whereas in the absence of it there was no reduction in 2 hours. No reduction occurred in the presence of DPN, in confirmation of the TPN specificity of this enzyme in plants (Euler *et al.*, 1939).

*d. α-Ketoglutarate. Iso*citric acid is oxidized through the intermediate formation of oxalosuccinic acid to α-ketoglutaric acid. Although no increase in oxygen uptake was noted on addition of 0·005 M. α-ketoglutarate, relatively vigorous utilization (Table V) occurred. It is possible that the lack of increase in O_2 uptake is due to the presence of oxidizable substrates in the stem of these plants. In fact, when the plants were starved for 4 days in the dark there was a 66 per cent. increase in O_2 uptake on addition of α-ketoglutarate (Fig. 7). This method of depleting plant tissues of respirable materials is superior to the drastic washing procedures now in vogue. Although the metabolic level of the plant is reduced, the leaching of necessary cofactors and regulators is avoided and damage to the semi-permeable membranes is minimized.

e. Succinate. The decarboxylative oxidation of α-ketoglutarate gives succinate. The presence of succinoxidase in tomato stem tissue was shown by measuring the O_2 uptake of pressed juice from tomato stems in the presence of succinate (2·1 ml. juice+0·6 ml. 0·2 M. phosphate buffer pH 7·4+0·3 ml. succinate, 0·1 M.) (Table V). Acetone powders of these tissues gave negative results.

Malonate may be considered a structural inhibitor of succinoxidase, an inhibitor which acts competitively and reversibly. Henderson and Stauffer (1944) reported no malonate inhibition of the O_2 uptake of cultured tomato root-tips at pH 5·8, and more recently Eberts *et al.* (1951) reported no malonate inhibition of the O_2 uptake of tomato stem slices previously washed for 15 hours and suspended in phosphate buffer, pH 5·5. These negative results are due to the cell membrane, for Turner and Hanly (1947) found that malonate penetrates the cell only as the undissociated acid. The effect of malonate (0·005 M.) on the O_2 uptake was therefore tested at different pH

values. Inhibition was definite at pH 3·5, whereas at pH 5·5 the inhibition was small (Table VI). Evidence that this inhibition is due to the competitive inhibition of succinoxidase was found by addition of 0·01 M. succinate to slices containing 0·005 M. malonate at pH 4·5. Under these conditions the inhibition was completely suppressed. Further, succinate was capable of completely reversing the effect of malonate.

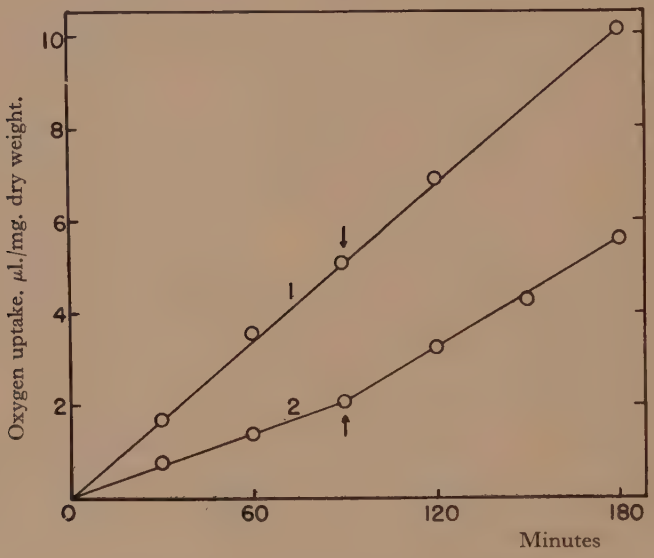

FIG. 7. Effect of 'starvation' on oxygen uptake increase following the addition of α-ketoglutarate 0·005 M., tipped 90 minutes after O_2 uptake measurements started (arrows). 1. Control. 2. 'Starved' plants

TABLE VI

Effect of pH *on the inhibition by malonate of the respiration of tomato stem slices*

Buffer, Teorell and Stenhagen's universal buffer. Malonate 0·005 M. Temp. 28°.

pH	Q_{O_2}	Q_{O_2} malonate	Inhibition %
3·5	2·2	1·3	41
4·0	2·2	1·65	25
4·5	2·3	1·5	34·7
5·5	2·45	2·2	10
6·0	2·2	2·15	2
7·0	2·1	2·05	0
8·0	1·9	1·9	0

f. Malate. The last oxidizable intermediate in the citric acid cycle is malic acid. Malate (0·01 M., pH 5·4) increased the O_2 uptake of tomato stem slices 25 per cent. (Table V). The oxidation of malate may take place either through

dehydrogenation to oxalacetic acid by malic dehydrogenase (Malate+DPN_{Ox} ⇌ Oxalacetate+$DPN_{Red}H$+H^+) or through decarboxylative oxidation by 'malic enzyme' (malate+TPN_{Ox} ⇌ Pyruvate+CO_2+TPN_{Red}+H^+). The presence of malic dehydrogenase was demonstrated in tomato stem homogenates in anaerobic experiments in the presence of DPN and ferricyanide as the oxidizing agents. The experiments were performed in Warburg manometers, the centre portion of which contained 1·0 ml. of homogenate; 0·5 ml. of water; 0·5 ml. 0·154 M. $NaHCO_3$; 0·3 ml. 0·2 M. neutralized cyanide; and 0·3 ml. 0·1 M. $K_3Fe(CN)_6$. The side arm contained 0·1 ml. DPN (100 μg.) and 0·3

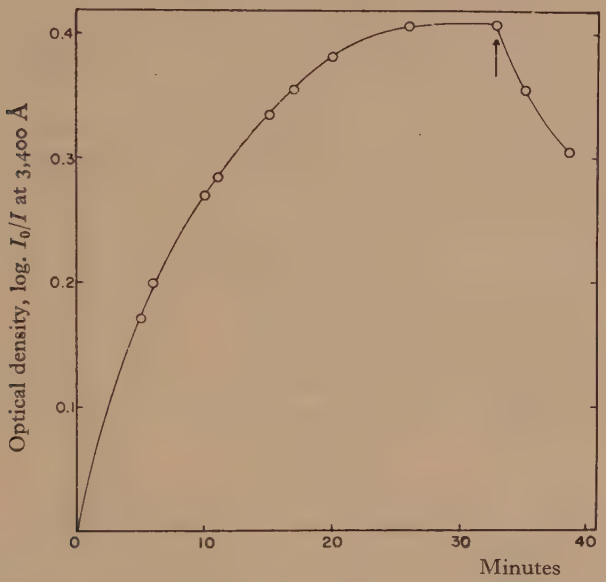

FIG. 8. Activity of the 'malic enzyme' of tomato stem tissues. Measured by the reduction of TPN on addition of malate. At point measured by arrow, pyruvate was added

ml. 0·1 M. malate or water. Fluid and gas phases were saturated with N_2:CO_2 (95:5). The large CO_2 formation in the control vessels must be due to oxidation of other metabolites present in the homogenate (Table V). The excess CO_2 uptake, 49·5, is equivalent to the oxidation of 24·7 μl. malate. The presence of Ochoa's 'malic enzyme' was found by following the methods of Vennesland *et al.*, which had demonstrated this enzyme in a large number of plants (Vennesland *et al.*, 1949; Conn *et al.*, 1949). Tomato stems were ground in a meat chopper, and the pulp and juice were pressed through cheesecloth. One half-volume of saturated $(NH_4)_2SO_4$ was added to the fluid which was centrifuged after 30 minutes. To the supernatant fluid obtained from this centrifugation another half-volume of saturated $(NH_4)_2SO_4$ was added; the suspension was centrifuged again after 30 minutes. The precipitate was dissolved in 0·01 M. phosphate, pH 7·4, and was dialysed overnight against 0·005 M.

phosphate buffer pH 7·4. Some precipitated protein was discarded after centrifugation. All operations were carried out at 3° C. The reactions were followed spectrophotometrically by measuring the reduction and oxidation of TPN at 3,400 Å in a Beckmann spectrophotometer. The quartz cuvettes contained: 1·0 ml. enzyme; 1·1 ml. glycylglycine buffer, pH 7·4; 0·3 ml. 0·05 M. $MnCl_2$; 0·1 ml. malate; and 0·4 ml. H_2O. At zero time 0·1 ml. of TPN (100 μg.) was added. Reversibility of the reaction was demonstrated by oxidation of reduced TPN on addition of 0·1 ml. pyruvate dissolved in 0·15 M. $NaHCO_3$ and saturated with CO_2 (Fig. 8).

g. Oxalacetate. Oxalacetate is formed by oxidation of malate, and is decarboxylated to pyruvate by oxalacetate decarboxylase. Its decarboxylation was followed manometrically. Pressed juice from tomato stems was mixed with one-half volume of neutralized saturated $(NH_4)_2SO_4$. After standing for 1 hour at 4° C., the suspension was centrifuged in the cold room in a Sorvall angle centrifuge at 5,000 r.p.m. The supernatant was treated with 0·25 volume of saturated $(NH_4)_2SO_4$. This was left standing at 4° C. for 1 hour and centrifuged at the same temperature. The precipitate was taken up in 0·025 M. phosphate, pH 7·4, and was dialysed overnight against 0·002 M. phosphate. The activity of this enzyme was rather low (Table V).

Oxidation by the malate pathway

The large accumulation of oxalic acid in some fungi and higher plants led Chrząszcz and Zakomorny (1933) to postulate the following sequence of reactions:

$$\text{Fumarate} + 2H_2O \rightarrow 2 \text{ glycolate} \xrightarrow{-2H_2O-2CO_2} 2 \text{ formate} \xrightarrow{-H_2} \text{oxalate.}$$

This pathway of oxidation, which may end with the formation of CO_2 and H_2, has been discussed by Barron (1951), who postulated that the oxidation may start from malate as indicated in the scheme below.

$$\text{Malate} \overset{+H_2O}{\rightleftharpoons} 2 \text{ glycolate} \overset{-2H_2}{\rightleftharpoons} 2 \text{ glyoxylate}$$

$$2 \text{ formate} \begin{cases} \xrightarrow{-H_2} \text{oxalate} \\ \xrightarrow{+O_2-2CO_2} 2CO_2 + H_2 \end{cases}$$

In this scheme the malic pathway of oxidation starts with the hydrolytic cleavage of malate into two molecules of glycolic acid. The enzyme 'malic hydrolase' seems to be present in the tomato stem. Tomato stem slices (hypocotyl and first internode tissue) were incubated in the dark in the presence of 100 μM. malate in Ringer-phosphate, pH 5·0, with N_2 as the gas phase to avoid oxidation of malate. Glycolic acid was determined at the end of 2 hours. 5·4 μM. of glycolic acid were produced.

Glycolic acid (0·005 M., pH 6·5) increased the O_2 uptake 23 per cent. (Table VI). On addition of aldehyde-fixing agents, such as semi-carbazide or

hydroxylamine, a 50 per cent. inhibition of glycolic acid oxidation was observed. Glyoxylic acid (0·005 M.) markedly increased the O_2 uptake of tomato stem slices (Table VII). These reactions had been studied in detail for higher plants by Tolbert *et al.* (1949).

The presence of formic dehydrogenase in tomato seeds was reported by Davison (1951). It is also present in tomato stem. The enzyme was extracted by grinding the tissue in 0·1 M. K_2HPO_4. One ml. of this filtered fluid was added to Thunberg tubes which contained 1 ml. 0·01 M. phosphate buffer, pH 7·0; 1 ml. 0·001 M. methylene blue; and 1 ml. 0·1 M. formate. The side bulb contained in 0·5 ml. of water 0·5 mg. DPN and 0·25 mg. ATP brought to pH 7·0 with NaOH. (The ATP was added to protect DPN from destruction by the nucleosidases, which are very active in plants.) The tubes were evacuated and the DPN–ATP mixture was tipped in. Complete reduction of the dye occurred in 6 minutes, whereas in the absence of formate reduction took 1 hour. In tomato stem slices addition of formate increased the O_2 uptake. There was concomitant utilization of formate (Table VII).

TABLE VII

The malic acid pathway in tomato stem slices

Buffer, Ringer-phosphate, pH 5·3. Substrate concentration 0·001 M.

Substrate	Q_{O_2}	Q substrate
Control	2·97	—
Malate	3·64	—
Glycolate	3·75	0·8
Glyoxylate	8·50	—
Formate	3·52	3·01

Nitrogen and fat metabolism

A few experiments were performed on the oxidation of amino acids and fatty acids. Added amino acids, with the exception of alanine, induced a definite increase in the O_2 uptake of tomato stem slices although no extra ammonia formation was observed. These findings are similar to those for potato tuber slices (Barron *et al.*, 1950) (Table VIII). Leonard and Burris (1947) reported transamination of glutamic and oxaloacetic acid in tomato stem tissues.

Addition of butyrate increased the O_2 uptake of tomato stem slices. In these experiments butyrate labelled with C^{14} in the carboxyl group was used. $C^{14}O_2$ was found in the respiratory CO_2, evidence of oxidation of butyrate, further confirmed by titration of the acid obtained after steam distillation. Attention is called to the considerable dilution of radioactive butyrate at the end of the experiment, evidence for the formation of butyrate in the tissue during the incubation period (Table IX).

TABLE VIII

Metabolism of amino acids and amides by tomato stem slices

Buffer, Ringer-phosphate, pH 5·4. Substrate concentration, 0·01 M. Temp. 28° C. Incubation, 2 hours. Q values give μl./mg. dry weight per hour.

Substrate	Q_{O_2}	Q_{NH_3}
None	1·76	0·145
Glutamic acid	2·19	0·063
Alanine	2·03	0·550
Aspartic acid	1·87	0·110
Glutamine	1·90	—
Asparagine	1·84	—

TABLE IX

Metabolism of butyrate by tomato stem slices

Buffer, Ringer-phosphate, pH 5·3, 5 ml., $CH_3CH_2CH_2C^{14}OONa$, 50 micromoles. Dry weight of slices, 59 mg. Incubation time, 2 hours.

Substance analysed	Butyrate utilization or O_2 uptake μl.	Counts/minute
O_2 uptake no substrate	339	—
O_2 uptake butyrate	370	—
Butyrate utilization	118	—
$CH_3CH_2CH_2C^{14}OONa$, initial	—	85, 580
$CH_3CH_2CH_2C^{14}OONa$, final	—	47, 928
$C^{14}O_2$ respiratory	—	810

Terminal oxidation

Caldwell and Meiklejohn (1937*b*) reported that cyanide and azide strongly inhibited respiration of slices of the tomato stem. This was confirmed by the finding that 0·005 M. cyanide inhibits this respiration 97 per cent. and that 0·01 M. and 0·001 M. azide inhibit it 94 per cent. and 81 per cent. respectively, and indicates that the terminal respiration of the tomato stem is mediated by enzyme systems with heavy metal prosthetic groups. The ready browning of cut tomato tissues indicates the presence of tyrosinase, whose activity in tomato stems was determined by Nagy *et al.* (1938).

Using carbon monoxide inhibition with intermittent illumination Link (1941) found that in tomato stem slices both iron and copper enzymes and one or more other enzymes (designated 'residual') may be operative in the transfer of electrons from substrates to molecular oxygen. These unpublished findings were corroborated by Link and Klein (1951).

Since azide and cyanide completely inhibit the 'residual' oxidase system or systems, it is probable that they too have heavy metal prosthetic groups. Tsui (1949), on the basis of gas-exchange rates of zinc-deficient and control tomato plants, reported that zinc-containing enzymes play a role in mediating oxygen uptake in terminal oxidation of these plants. His data are not convincing

because addition of 0·01 M. Zn did not restore oxidative activity to slices inhibited by 0·005 M. cyanide.

Naito and Isamaru (1938) reported the presence of ascorbic acid oxidase in the tomato stem. The oxygen uptake of tomato stem slices supplied with 0·001 M. ascorbic acid was completely inhibited by 0·005 M. cyanide. Ascorbic acid itself was not oxidized.

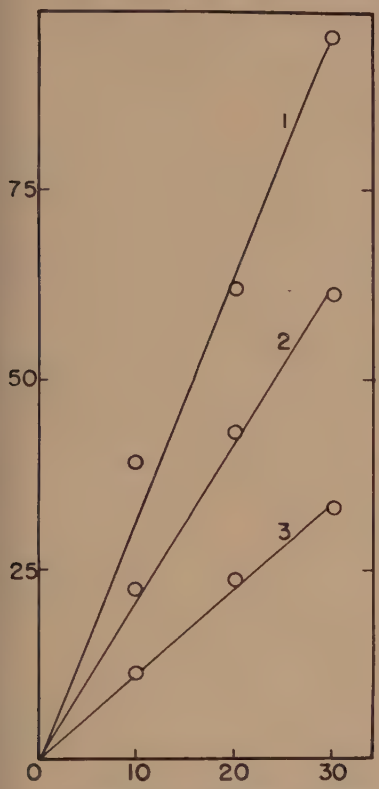

FIG. 9. Assay for cytochrome oxidase of fresh tomato stem tissue press juice. Each vessel contained 0·5 ml. PO_4, 0·2 M., pH 7·4; 0·6 ml. cytochrome *c*, 0·0004 M.; 0·15 ml. $AlCl_3$, 0·008 M.; 0·3 ml. ascorbic acid, 0·1 M. Final volume, 2·0 ml. Temp. 28° C. 1. 1·35 ml. juice. 2. 0·90 ml. juice. 3. 0·45 ml. juice. Ordinates, oxygen uptake, μl. Abscissae, minutes

8-hydroxyquinoline had little effect on the oxygen uptake of tomato stem tissues and had no direct effect on the oxidation of catechol or *p*-cresol by purified mushroom tyrosinase. In potato slices this heavy metal-complexing agent inhibited respiration and the oxidation of catechol (Barron *et al.*, 1950).

The dissociability of the Cu-tyrosinase complex in tomato stem tissues was demonstrated as follows. Oxygen uptake of slices was measured in the presence of cyanide followed by a 30-minute leaching period with three changes of 0·01 M. PO_4, pH 5·3. The increase in O_2 uptake after washing can be ascribed to dissociation of the Fe-CN complex (Warburg, 1924). Since Kubowitz (1938) had shown that the Cu-CN complex of mushroom and potato tyrosinase is not easily dissociated it is reasonable to suppose that the copper prosthetic group of tyrosinase had been washed out. Copper sulphate (0·0001 M.) was then added to control and washed tissue and further significant increase of O_2 uptake was observed only in the washed slices. This demonstrates the applicability to tissues of higher plants of the Warburg and Kubowitz dissociation techniques.

Because all experiments designed to determine which enzyme systems may transfer electrons from substrate to molecular oxygen by inhibition techniques are open to criticism, direct proof of the presence and activity of potential terminal oxidase systems was sought. Cytochrome oxidase was found in fresh press juice of tomato stems as well as in acetone powders of the juice. Steady rates of O_2 uptake, directly proportional to the amount of enzyme added, were demonstrated for each preparation (Fig. 9).

The ability of polyphenol oxidase to act as a link in the electron transfer from substrate to oxygen was tested directly by using a modification of the

coupled oxidation-reduction reported for tyrosinase by Kubowitz (1938). He demonstrated this possibility for the oxidation-reduction systems hexose-phosphate ⇌ phosphohexonic acid catalysed by TPN, and quinone ⇌ hydroquinone catalysed by Cu-protein:

$$\text{Hexose phosphate} + \text{TPN} \rightarrow \text{phosphohexonic acid} + \text{TPNH}_2$$

$$\text{TPNH}_2 + \text{quinone} \rightarrow \text{TPN} + \text{hydroquinone}$$

$$\text{Hydroquinone} + \text{Cu}^{++}\ \text{(tyrosinase)} \rightarrow \text{quinone} + \text{Cu}^{+}\ \text{(tyrosinase)}.$$

Although these findings have been widely used in postulation of the participation of tyrosinase in plant respiration, no direct test of the presence of such coupled reactions in tissues of higher plants has been made. The existence of direct electron transfer from substrate to oxygen via tyrosinase was tested in homogenates of tomato stem tissue by incubating the tissue suspension in the presence of α-ketoglutarate; of α-ketoglutarate+DPN; and of α-ketoglutarate, catechol, and DPN (Table X). Only the last vessels showed definite oxidation of α-ketoglutarate, evidence that this substrate was oxidized *via* tyrosinase according to the following reactions:

$$\alpha\text{-ketoglutarate} + \text{DPN} \rightleftharpoons \text{succinate} + \text{DPNH}_2$$

$$\text{DPNH}_2 + \text{quinone} \rightleftharpoons \text{catechol} + \text{DPN}$$

$$\text{Catechol} + 2\ \text{Cu}^{++}\ \text{(tyrosinase)} \rightleftharpoons \text{quinone} + 2\ \text{Cu}^{+}\ \text{(tyrosinase)} + 2\text{H}^{+}$$

$$\text{O}_2 + 2\text{H}^{+} \rightarrow \text{H}_2\text{O}_2 \qquad \text{H}_2\text{O}_2 \rightarrow \text{H}_2\text{O} + \tfrac{1}{2}\text{O}_2$$

TABLE X

The oxidation of α-ketoglutarate by the catechol-tyrosinase system

Tissue homogenate, 2 ml.; Ringer-phosphate pH 7·0, 1 ml.; α-ketoglutarate, 50 μM., 0·3 ml.; catechol, 0·17 μM., 0·1 ml; DPN, 0·1 μM., 0·1 ml; Duration of experiments 60 minutes, temp. 28° C.

Experimental conditions	O_2 uptake μl.	α-ketoglutarate utilization μl.
Tissue homogenate . . .	0	0
Tissue+catechol. . . .	0	0
Tissue+α-ketoglutarate . .	0	0
Tissue+α-ketogl.+catechol . .	10	0
Tissue+α-ketogl.+DPN . .	0	0
Tissue+α-ketogl.+DPN+catechol	55·5	2·7

The discrepancy between the O_2 uptake and the α-ketoglutarate utilization may be due to other oxidations proceeding through the same route.

These experiments demonstrate for the first time that electron transfer from DPN to oxygen via polyphenoloxidase can actually be made to occur in homogenates of tissues of a higher plant, and indicate that it might occur in the tissues of the intact tomato stem.

DISCUSSION

The respiration of the germinating tomato seed through seedling development was studied by Bartlett (1942). He found that the early stages of seedling respiration are characterized by oxidative processes which shift from an incomplete fat oxidation (R.Q. 0·3) upon germination to a complete fat oxidation (R.Q. 0·7) in 5- to 7-day-old seedlings. By the tenth day after germination the respiratory quotient rises to 1·0 (Klein, 1952). This respiratory quotient of 1·0 seems to prevail in the stem tissue (as shown in the experiments presented here and in those of Caldwell and Meiklejohn (1937*a*)), as well as in the leaves (Klenker, 1950). The great variation noted in the Q_{O_2} values of tomato stem slices is due not only to age differences (Caldwell and Meiklejohn, 1937*a*) but probably also to the nutritional status of the plant. Plants of the same age had a higher Q_{O_2} value when in a good nutritional state.

The anaerobic carbohydrate fermentation in animal tissues is glycolytic (lactic acid fermentation), whereas in yeast it is alcoholic (alcohol fermentation). Barron *et al.* (1950) found both types of fermentation in potato tuber disks, alcohol fermentation being 17 times higher. In tomato stem slices also, anaerobic carbohydrate metabolism ended in glycolysis and alcohol fermentation; however, the preponderance of alcohol fermentation was much lower than in potato, the ratio of alcohol:lactic acid being only 6·6:1. Alcohol and acetaldehyde have been found in tomato fruit (Gustafson, 1934), but the presence of glycolytic fermentation in the fruit has not been reported.

The glycolytic and alcoholic fermentations in the tomato stem slices, as in potato tuber slices, seem to occur according to the Embden–Meyerhof scheme, which starts with the phosphorylation of starch or sugar, and continues in a series of reactions catalysed by thirteen specific enzymes (Barron, 1943). Since the primary carbohydrate reserve in tomato slices is preponderantly sugar, phosphorylation in these tissues begins with sugars. The data presented in this paper favour the view that carbohydrate fermentation in tomato stem slices occurs through the same series of enzymes. In contrast to animal tissues or yeast, the terminal enzymes, lactic dehydrogenase and alcohol dehydrogenase, are both present in tomato stem and in potato tuber slices.

The anaerobic phase of carbohydrate metabolism in tomato stem slices ends with the formation of 75 per cent. ethyl alcohol and 13·7 per cent. of lactic acid. Since neither alcohol nor lactic acid is found in aerobiosis, the oxidative phase must start with the oxidation of acetaldehyde and/or of pyruvic acid. Which of these oxidations is the preferred pathway is not known. When pyruvic acid *was added* to tomato stem slices it was preferentially oxidized to acetate, as shown by the presence of acetic acid and by its accumulation on addition of fluoroacetate. The fate of added pyruvate was similar to that observed by Barron, Ardao, and Hearon (1950) in bakers' yeast, i.e. utilization by direct oxidation in spite of the presence of carboxylase, which could have decarboxylated it to acetaldehyde. Possibly we are confronted in tomato stem tissues with the same problem as in yeast cells, namely, differential

topographic distribution of enzymes, carboxylase being at the centre of the cell and pyruvate oxidase at its periphery.

It has been inferred on the basis of inhibition studies and analogy that the oxidative phase of carbohydrate metabolism in tissues of higher plants occurs via the citric acid cycle (see Bonner, 1950). The first experimental demonstration, however, of the synthesis of citric acid from acetic acid and oxalacetic acid—the first step in the cycle—was not made until Barron *et al.* (1950) reported this reaction for potato tuber slices. This condensation of acetate with oxaloacetate to give citrate also was demonstrated in tomato stem tissue. Furthermore, the presence of *iso*citric dehydrogenase and of aconitase provide further support for the hypothesis that the oxidative pathway of carbohydrate in tomato stem also is via the citric acid cycle. The presence of succinoxidase and the inhibition of the respiration, as well as of the oxidation, of pyruvate, of acetate, and of succinate by malonic acid are further evidence that carbohydrate in tomato stem slices may be oxidized via the citric acid cycle. The lack of inhibition of respiration by malonate reported by Eberts *et al* (1951) was probably due to failure by these investigators to take into consideration the fact that the cell membrane is impermeable to the dissociated salts of organic acids. The experiments presented in this paper on increase of malonate inhibition and on increase of pyruvate utilization with decrease of pH are evidence for this contention.

The malic acid pathway seems to be possible as an accessory route of respiration in tomato stem slices. Malic acid was split to glycolic acid anaerobically, and glycolic, glyoxylic, and formic acids were found to be oxidized. The presence of formic dehydrogenase in tomato stem is further evidence for the possibility of occurrence of this pathway (cf. Davison, 1951). Whether the first step is a simple hydrolytic splitting or a more complicated reaction remains to be determined.

The utilization and oxidation of butyric and acetic acids by tomato stem slices, together with the evidence of new formation of these acids in those experiments in which dilution of C^{14}-labelled compounds was found, demonstrate that an active fatty acid metabolism does exist in these tissues. In spite of this active fatty acid metabolism, a respiratory quotient of one was found. It may be concluded from these experiments that R.Q. values alone are no proof for a single pathway of oxidation, be it carbohydrate, fat, or protein oxidation.

The problem of the terminal system in plants for electron transfer to molecular oxygen created by the experiments of Kubowitz (1938) on coupled oxidation–reduction of TPN and polyphenol oxidase-catalysed reactions has been unduly complicated in the past by the exaggerated importance given to inhibition experiments. Cytochrome–cytochrome oxidase and the polyphenol oxidase systems seem to exist in most plant tissues. Since both are heavy-metal-conjugated proteins their clear differentiation by inhibitors is difficult. In tomato stem the respiration is partially inhibited reversibly by CO (evidence that cytochrome oxidase takes part in the respiration) and by heavy-metal

inhibitors (indication that both systems *may* take part in respiration). An experimental demonstration for tomato stem tissues of the coupled oxidation–reduction postulated by Kubowitz was attempted by using α-ketoglutarate as the oxidizable substrate coupled with tyrosinase present in the tissue and added catechol. In homogenized tomato stem tissue, α-ketoglutarate was rapidly oxidized by atmospheric oxygen only in the presence of DPN and of catechol, a demonstration that electron transfer may take place in these tissues via polyphenol oxidase. The necessity for DPN addition must be due to the great activity of nucleosidases in plant tissues, which rapidly destroy the cellular pyridine nucleotides when the tissues are ground. It must be emphasized, however, that these experiments, as well as the inhibition experiments, do not tell us the actual path of electrons to molecular oxygen. They only show that both pathways are possible and that they may function under appropriate conditions. The relative importance of the cytochrome–cytochrome oxidase system, of the polyphenol oxidase system, and of other systems indicated to be present in tomato stem tissues will be determined by the topographical distribution of these enzymes, their relative abundance, and the operation of regulatory mechanisms as yet unknown.

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The Path of Carbon in Photosynthesis

XIII. pH EFFECTS IN $C^{14}O_2$ FIXATION BY *SCENEDESMUS*[1]

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SUMMARY

The rates of the photosynthetic and dark fixations of $C^{14}O_2$ in *Scenedesmus* have been compared in dilute phosphate buffers ranging from pH 1·6 to pH 11·4, and the amounts of carbon incorporated into the various products have been determined by means of the radiochromatographic method.

In photosynthesis, an acid medium favours early incorporation of C^{14} into sucrose, polysaccharides, and the three-carbon compounds alanine and serine. Fixation into the four-carbon compounds malic and aspartic acids is enhanced in an alkaline medium. Kinetic experiments at several pH values suggest that several paths may be available for carbon dioxide assimilation.

A tentative correlation of the results with the pH optima of some enzymes and resultant effects upon concentrations of intermediates is presented.

THE extent to which the internal pH of a living cell can be influenced by that of the external medium is still a matter of uncertainty. Since the enzymes involved in the various metabolic processes have different pH optima, it may be expected that a shift in the internal pH would exert a selective influence upon the rates of those processes. Effects of this kind, coupled with changes in oxidation-reduction potentials, may well be related to the differences between the paths of carbon in photosynthesis, dark assimilation, and respiration. Since many of the features of the photosynthetic assimilation of radioactive carbon dioxide have now been revealed (Benson and Calvin, 1950; Benson, Bassham *et al.*, 1950; Calvin *et al.*, 1950), it seemed to us that this reaction might provide a useful tool for the study of internal pH effects and that these, in turn, could contribute to our understanding of the various steps involved in photosynthesis.

The effect of pH upon the over-all rate of photosynthesis has been the object of rather few systematic investigations (Rabinowitch, 1945; Small, 1946), although a number of incidental observations are scattered through the literature. Emerson and Green (1938) found no appreciable variation between pH 4·6 and 8·9 in *Chlorella*, Pratt (1943) reported large differences in more alkaline solutions over long periods, and Brilliant (1949) observed differences in a number of species. Recently Warburg and Burk (1950) measured higher quantum yields at pH 5 than at pH 9, and Franck (1949) postulated an influence of pH upon the permeability of the chloroplast membrane.

Factors such as membrane permeability, the concentration of carbon

[1] The work described in this paper was sponsored by the U.S. Atomic Energy Commission.

[2] Guggenheim Fellow 1949, on leave from Laval University, Quebec, Canada.

dioxide and its distribution among the species H_2CO_3, HCO_3^-, and CO_3^{--}, and also the value of the photosynthetic quotient, are difficult to control and to separate from a direct influence of pH changes upon the metabolic steps. In this preliminary and forcibly qualitative survey, no strict control of the above variables could be attempted. In order to minimize the action of transition and adaptation phenomena, the cells were exposed to the buffer for identical periods before each $C^{14}O_2$ fixation experiment. Ten minutes was found to be sufficient for the buffer to exert its full effect upon the rate of fixation of carbon dioxide and is believed to be too brief to permit the changes in enzyme constitution by means of which growing cells adapt themselves to a new medium.

EXPERIMENTAL METHODS

About 1 litre of a 1-day growth from a continuous culture of *Scenedesmus* was freed from the culture medium by centrifugation and resuspended in distilled water at a concentration of 1 ml. wet-packed cells in 100 ml. A stream of air was passed through this suspension for 1 to 3 hours in subdued daylight at room temperature. Experiments in a series were carried out with 20-ml. aliquots (0·2 c.c. cells) from the same suspension. The samples were placed in a flat, circular vessel of about 5 mm. internal thickness, held in a vertical position and illuminated from both sides. The algae were first allowed an adaptation period of 10 minutes in the light in the presence of M/300 phosphate buffer of the chosen pH while air streamed through the suspension at the rate of about three bubbles per second. The radioactive sodium bicarbonate solution (generally about 10 microcuries, 0·001 millimole) was then injected; the flask was stoppered at once and shaken throughout the photosynthetic fixation period. The cells were then killed in boiling ethanol, either directly or after rapidly filtering the suspension with Celite filter aid. Paper chromatography of the 80 per cent. ethanol extract necessitated the previous removal of the salts. The extracts were analysed by means of the radiochromatographic technique (Benson, Bassham *et al.*, 1950).

In order to avoid possible specific effects resulting from the use of different anions, phosphate buffers were used over the whole pH scale in spite of their low buffering power in certain regions. Blank experiments, in which non-radioactive sodium bicarbonate or carbonate was injected, showed that the pH of the buffered cell suspensions, at various acidities, did not vary by more than 0·1 pH unit during the period of adaptation and photosynthesis.

The effects of pH on the concentrations of some of the intermediates of photosynthesis were determined for two pH values in the following manner. A suspension of 1 g. of *Scenedesmus* was illuminated for 20 hours in the presence of nutrient solution (pH 6), 5 per cent. carbon dioxide in air, and 0·5 mc. radiophosphate. Identical aliquot portions were adjusted to pH 2 and to pH 10·6 with hydrochloric acid and sodium hydroxide. After 30 minutes' photosynthesis at moderate light intensity with excess 4 per cent. carbon dioxide in air, the samples were quickly centrifuged and killed in hot ethanol.

The amounts of labelled phosphoglycerate and hexose monophosphates, separated chromatographically, were compared by direct counting of the radioactive areas.

The total phosphoglycerate radioactivity in the extract was 2·5 times as great at pH 10·6 as at pH 2. The corresponding ratio of activity in the hexose (largely glucose) monophosphates was 1·7.

EXPERIMENTAL RESULTS

Influence of pH on the fixation rate. In preliminary experiments it was found that, after standing 30 minutes at pH 1 or 11·5, the algae were still able to fix

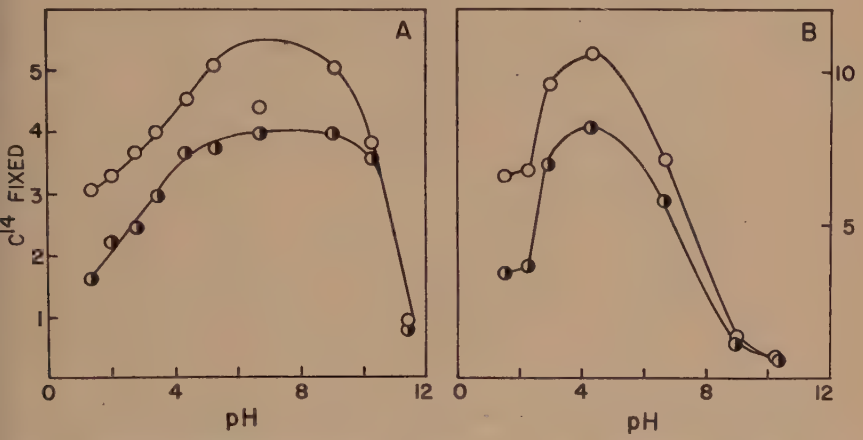

FIG. 1. Total and soluble radioactivity, in millions of counts per minute per gram cells, fixed in 2 minutes' photosynthesis at light intensities of (A) 2,500 foot-candles and (B) 5,000 foot-candles. ○ total; ◑ soluble

appreciable amounts of $C^{14}O_2$ in the light. Exposure to those media for various lengths of time showed that the fixation rates decreased during the first 5 to 10 minutes and thereafter remained constant for at least another 20 minutes. When such an acid or alkaline suspension was brought back to neutrality 10 minutes before the fixation experiment, the rate was the same as in a sample of the original neutral suspension, indicating that the rate-depressing effect of exposure to an extreme pH is fully reversible over the brief periods used in this work. All experiments were therefore carried out after a 10-minute exposure of the cells to the buffer.

Typical curves illustrating the influence of pH on the fixation rate are shown in Fig. 1. In seven experiments with different crops of algae the position and shape of the maximum exhibited considerable variations, Fig. 1A being representative of the average trend and Fig. 1B illustrating an extreme case. It is not known to what extent the deviations are due to differences between the properties of the crops or to the imperfect control of the experimental conditions, especially the concentration of carbon dioxide. There is no clear correlation with light intensity. The most notable feature is the absence of

insoluble radioactive compounds in all experiments in strongly alkaline media and their generally high proportion on the acid side. Near pH 3, a slight minimum in the fixation rate was observed in several experiments, but the reasons for this (possibly artificial) effect are not yet clear. It was also noted that algae which had photosynthesized below pH 3 yielded yellow alcoholic extracts, possibly due to pheophytization of the chlorophyll.

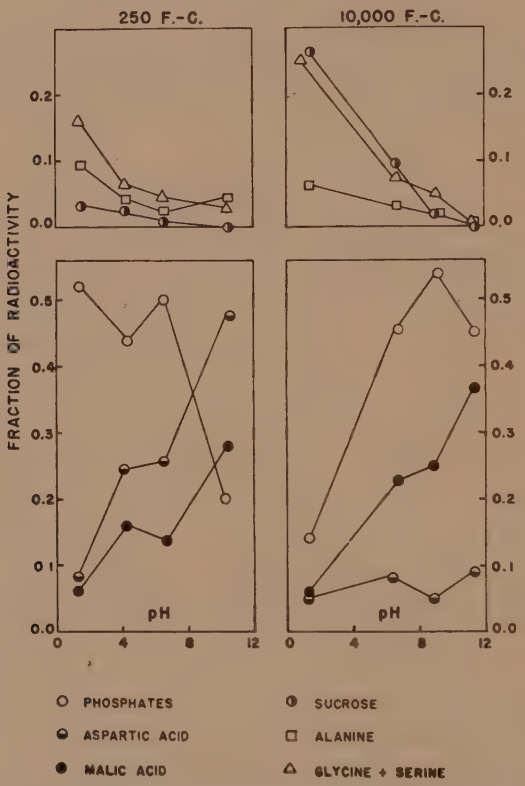

FIG. 2. Fractions of soluble radioactivity in the labelled intermediates after 2 minutes' photosynthesis at 250 and 10,000 foot-candles and at various pH values, in M/300 phosphate buffer

Distribution of labelled intermediates. Large differences were found in the relative radioactivity of labelled intermediates resulting from 2-minute photosyntheses in acid or alkaline media. The trends shown in Fig. 2 for two experiments at the lowest and highest light intensities used were qualitatively the same in all the other experiments, in spite of the difference in total fixation rates.

With increasing pH, the fraction of the radioactivity fixed in the four-carbon compounds malic and aspartic acids during 2 minutes increases while the fraction in the three-carbon compounds alanine and serine, and also in sucrose, decreases. The fraction in polysaccharides (insoluble material) also

decreases. The activity incorporated into the pentose, hexose, and heptose monophosphates seems to depend strongly upon the light intensity; on the whole, it increases with pH at the higher intensities but decreases at low intensities, two extreme cases being shown in Fig. 2. The pH dependence of malic acid is the same over a wide range of conditions, as shown by the curves in Figs. 2 and 3, which also suggest the existence of a secondary maximum near pH 4.

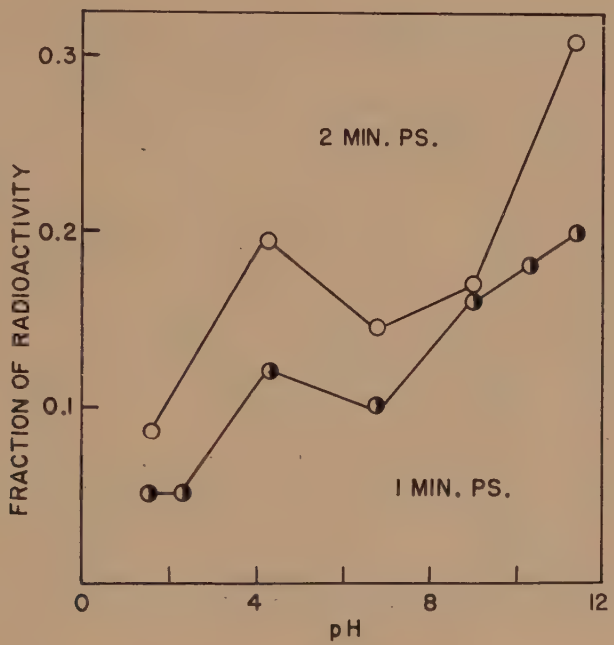

FIG. 3. Fractions of the soluble radioactivity in malic acid at various pH values after 1 minute photo-synthesis at 2,500 foot-candles and 2 minutes at 3,500 foot-candles

Kinetic experiments (Calvin *et al.*, 1950; Badin and Calvin, 1950) have already indicated two possible modes of initial incorporation of carbon dioxide: one into a three-carbon compound and one into a four-carbon compound. From our results it seems that an acid medium accelerates initial incorporation into three-carbon compounds and an alkaline one accelerates four-carbon compound synthesis. Further support for this hypothesis is found in the curves of Fig. 4. At the initial instant of an experiment the compound (or compounds) into which carbon dioxide is fixed should contain the totality of the radioactivity. The fraction of total activity in such compounds should decrease as more and more C^{14} is passed on to subsequent intermediates. This behaviour is exhibited by alanine at both pH 1·6 and 10·3 and by malic acid at pH 10·3. The time series does not extend to short times and recent experiments in this laboratory have shown that the activity fraction in alanine decreases to zero in very short photosyntheses. The phosphate curves, of

which phosphoglycerate is the major component, represents the principal point of C^{14} entry on the acid side but not on the alkaline one. The curves for serine and sucrose (not shown in the diagrams) are almost exactly complementary to the fluctuating curve for phosphate at pH 1·6, whereas at pH 10·3 serine and sucrose start from zero and increase very slowly to a value of about 5 per cent. after 5 minutes.

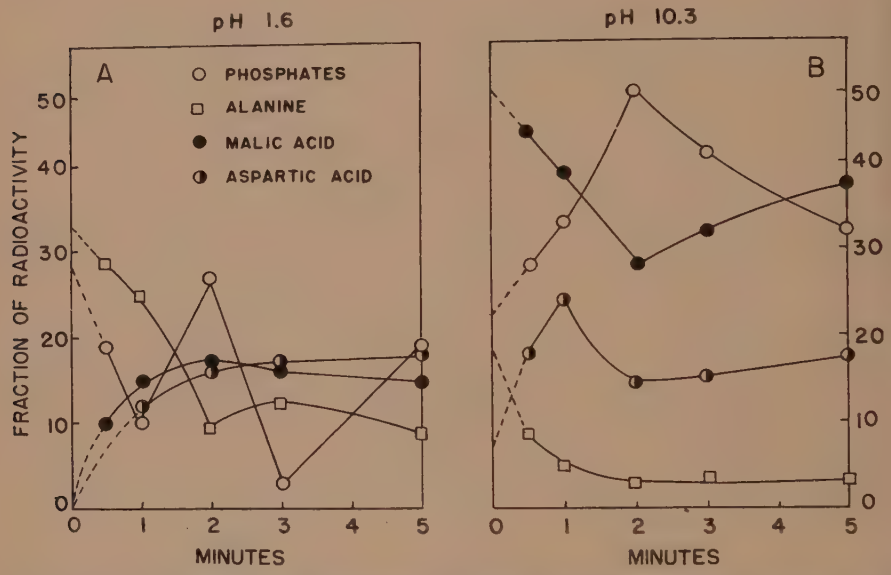

FIG. 4. Fractions of the soluble radioactivity in some of the intermediates at various times during photo-synthesis at (A) pH 1·6 and 9,000 foot-candles, and (B) pH 10·3 and 7,500 foot-candles

In view of a possible effect upon the distribution of labelled intermediates resulting from the action of the phosphate ions contained in the buffer, a series of 1-minute photosynthesis experiments was carried out in pH 6·7 buffer at four external concentrations ranging from zero (washed cells) to M/1000. The result was a 50 per cent. increase in the over-all fixation rate at the higher concentration, without notable change in the proportions of the labelled intermediates.

Dark fixation. It is known (Benson and Calvin, 1950) that the labelled products of the fixation of carbon dioxide in the dark differ from those of photosynthesis mainly by the absence of insoluble materials, phosphates and sucrose, and by the predominance of several of the tricarboxylic acid cycle intermediates. In two dark fixation experiments the effect of pH upon the total fixation rate was essentially the same as in photosynthesis, except for the absence of insoluble radioactive products. Fig. 5 shows the distribution of the labelled intermediates after 90 minutes' fixation in the dark. Of the compounds common to both dark and light fixations, alanine shows the same behaviour as

in the light, but malic acid, while still exhibiting a maximum near pH 4, no longer shows the large increase at high pH which may be a characteristic of photosynthesis. It is seen that the pH also exerts considerable influence upon the proportions of the labelled compounds more specifically connected with the dark assimilation.

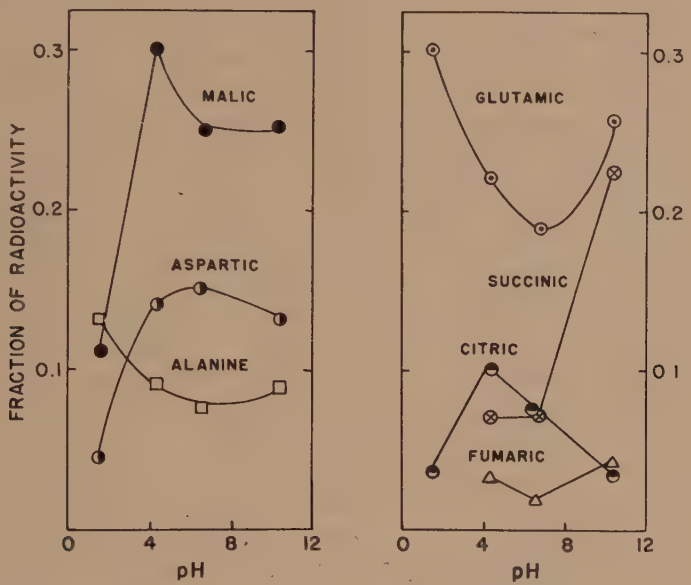

FIG. 5. Fractions of the radioactivity in the intermediates after 90 minutes' dark assimilation at various pH values

DISCUSSION

The large changes observed in the distribution of the labelled intermediates show the extent to which the metabolic régime is altered by a shift in the pH of the medium. It is impossible to estimate the relative importance of a number of pH-sensitive factors such as the carbon dioxide concentration, variations in the intracellular pH, localization of certain key steps at the membrane or self-regulating mechanisms which induce the production of the stronger C_4 acids in an alkaline medium and that of the weaker C_3 acids and neutral sucrose in an acid medium.

It seems that there are at least two initial reactions by which carbon dioxide can enter the system. The first, which predominates on the acid side, leads to organic phosphates of which the earliest component to appear has been shown (Badin and Calvin, 1950) to be phosphoglyceric acid. The second reaction leads to malic acid and predominates in strongly alkaline media. The use of an extreme pH seems to provide a means of studying each of these reactions with a minimum of interference from the other.

Assuming, as a working hypothesis, that the observed effects are due mainly

to changes in rate constants of the various enzymatic reactions brought about by shifts in intracellular pH, it would be of interest to correlate the observations with the pH optima of known enzymes. Those involved in carboxylations have optima around pH 4–6, the dehydrogenases near pH 9; several glycolytic enzymes show optima in the region of pH 8–9. The β-carboxylation reaction forming malic acid has an optimum near pH 5. Whether malic acid will accumulate or not depends primarily upon the relative rates of formation, its subsequent conversion to aspartic acid, and its reduction to unknown intermediates. If high pH reduces the rate constant for reduction of C_4 compounds, the result would be an increase in concentration of C_4 compounds. The conversion of a C_3 carbon dioxide acceptor to malic acid might have an optimum rate around pH 5 or pH 9 according to whether the carboxylation or subsequent hydrogenation is the rate-limiting step under the experimental conditions.

The pH optimum for sucrose phosphorylase of *Pseudomonas saccharophila* (Hassid, Doudoroff, and Barker, 1944) is about 6. At higher pH the equilibrium favours the cleavage of sucrose. The greater proportion of the C^{14} fixed in insolubles, largely polysaccharides, on the acid side seems to be in accord with the rather flat optima at pH 4–6 of the amylases. The precursors of the polysaccharides are also those for sucrose synthesis. The effect of low pH in increasing both sucrose and polysaccharide synthesis is therefore quite reasonable.

However, in the absence of more information on the magnitude of the lag between the intracellular and extracellular pH, on the value of the oxidation-reduction potential in the cell during photosynthesis, on the localization of the enzymes, and in view of the general complexity of the metabolic network, it is clear that the type of correlation attempted above can only provide suggestions or at best circumstantial evidence in favour of any particular mechanism.

The concentration of a given intermediate in the steady state systems is a function of the rates of all the reactions associated with its formation and conversion. It is clear that the initial rate of accumulation of C^{14} in a given compound is directly dependent upon the rate of its formation from the labelled precursor. For this reason the rate of C^{14} accumulation is dependent upon the rates of synthesis and conversion or the concentration of each previous intermediate through which it has passed and upon rates of any side reactions or equilibrations involving other reservoirs such as M in the scheme.

$$C^{14}O_2 + A \xrightarrow{k_1} B \xrightarrow{k_2} C \xrightarrow{k_3} D \longrightarrow \text{Sucrose}$$
$$\Downarrow\Uparrow$$
$$M$$

The rate constant, k, of each reaction may be a function of pH as well as other variables. If k_1 were increased or if k_2 were decreased by high pH, B would accumulate, as would M (malic acid, for example), until a new steady state were established. Although the actual rate of synthesis of sucrose would

increase if k_1 increased and would decrease slightly if k_2 decreased, the increase in the concentration of B would so decrease the specific activity of B that the C^{14} reaching the sucrose reservoir could diminish greatly. It should be pointed out that appearance of labelled sucrose in *Scenedesmus* begins about 2 minutes after administration of C^{14} and that the observations presented in Fig. 2 represent nearly an optimum in sensitivity of labelled sucrose synthesis towards external conditions. The measurement of concentrations of intermediates preceding sucrose may provide a clue to the interpretation of the effects of external conditions on the accumulation of C^{14} in sucrose as a function of time. The effect of pH upon the concentration of phosphoglycerate in *Scenedesmus* (the reservoir of phosphoglycerate is 4 to 5 times larger than that of all the other phosphorylated intermediates in sucrose synthesis) can possibly account for the 10-fold increase in amount of radiosucrose synthesized at pH 2 during the initial 2 minutes in spite of the fact that several glycolytic enzymes have their optima at relatively high pH.

The authors express their appreciation to Professor Melvin Calvin and Dr. J. A. Bassham for their interest in this work.

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The Relation between the Structure of Coumarin and its Derivatives, and their Activity as Germination Inhibitors

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SUMMARY

A large number of substitution derivatives of coumarin were examined. In every case the activity was wholly or partially removed by substitution. A number of growth regulators of the 2:4-D type were examined. These acted as germination inhibitors, in some cases being more active than coumarin.

It is concluded that the activity of coumarin as a germination inhibitor is due to its specific structure, consisting of an unsaturated lactone linked to an unsubstituted benzene nucleus. Any change in this structure leads to the partial destruction of the activity as a germination inhibitor.

INTRODUCTION

COUMARIN has been long recognized as a germination inhibitor (Sigmund, 1914). It is of special interest both due to its effectiveness at low concentrations and due to its relationship to the problems of light sensitivity of seeds (Evenari, 1949).

The action of coumarin as an inhibitor has been studied by Audus (1949), Audus and Quastel (1947), and Veldstra (1943–5).

Bonner and Thimann (1949) suggest that coumarin acts by its effect on certain —SH containing enzymes. This is not fully supported by the work of Goodwin and Taves (1950).

The fundamental problem which none of these investigators have fully answered is whether the lactone structure, the unsaturated lactone structure, or the unmodified molecule of coumarin is responsible for the inhibitory action. It will be shown here that it is in fact coumarin in its unmodified form which is the only really effective germination inhibitor, among those studied here. The only substances which were found to be more active than coumarin were various growth-promoting substances of the 2:4-D type. From work carried out until now there is good reason to believe that these substances act entirely differently from coumarin (Mayer and Evenari, 1951).

METHODS

The compounds under investigation were dissolved in water and tested as germination inhibitors. The seeds were placed in Petri dishes covered with filter-paper, and 3 ml. of solution were added when lettuce seeds were investigated and 5 ml. in the case of wheat. These conditions gave optimum germination (95–100 per cent.) in the dark and at 26° C. In a few isolated cases

substances insoluble in water were dissolved in dilute alkali or acids, suitable controls being run simultaneously. Investigation has shown that between pH 3 and 10 the germination is normal, both in buffer solutions and in dilute acids or alkali.

Germination tests were made in duplicate Petri dishes containing about 100 seeds of lettuce or in triplicates of 60 seeds in the case of wheat. The Petri dishes were placed in closed light-proof boxes and kept in a darkroom at constant temperature.

From the data obtained of germination percentages at different concentrations graphs were drawn and the molar concentration giving 50 per cent. germination calculated. From this the inhibition index (I.I.) of the substance studied is obtained. This index gives the relation of the substance investigated to the inhibitory action of coumarin and is obtained by dividing the molar concentration of coumarin giving 50 per cent. germination by the molar concentration of the compound giving the same germination percentage. In a few cases the I.I. was calculated at higher germination values, but as the germination-concentration curves are generally straight-line relationships, over the range studied, this is of little importance. Where the I.I. is given as being below a certain limit (I.I. $= <$——) this indicates the activity of the substance at its maximum solubility.

The lettuce seeds were of the variety 'Progress' of the years 1949 and 1950 from Messrs. Pieters-Wheeler which gave 50 per cent. germination at a coumarin concentration of $0{\cdot}5 \times 10^{-3}$ M. The wheat which was obtained from Kinnereth (Israel) gave 50 per cent. germination at a coumarin concentration of $1{\cdot}5\text{–}3 \times 10^{-3}$ M, depending on the samples used.

The compounds investigated were mostly synthesized by the methods described in the literature. Some were received as gifts, and their sources are listed in the acknowledgements.

RESULTS

1. *Coumarin and its derivatives*

(*a*) *Lettuce.* The introduction of any group into the coumarin structure either in the benzene nucleus or in the lactone ring wholly or partially abolished its activity as a germination inhibitor. The inhibition indices are given in Table I.

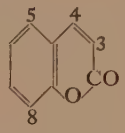
The introduction of two substituents completely destroys the activity of coumarin. The order of activity of a single substituent in removing the inhibitory action is $-NO_2 > -OH > -Cl$. Esterification of the hydroxyl groups does not in any way affect the lack of activity of the hydroxy-substituted coumarins. Slight modification in the coumarin structure, while retaining the stereochemical configuration, also sufficed to abolish its activity. Thus melilotic lactone (3:4-dihydrocoumarin) has a much reduced activity, while carbostyril (2-hydroxyquinoline) is entirely inactive.

Particularly striking is the fact that the halogenation of coumarin reduces its activity to a third or less. This is in marked contrast to the activity both as

inhibitors and growth promoters of the halogenated substances of the 2:4-D type.

TABLE I

Inhibition indices of coumarin and its derivatives



Compound	Inhibition Index		Compound	Inhibition Index	
	Lettuce	Wheat		Lettuce	Wheat
Dicoumarin	2·0	1·66	6:7-dihydroxycoumarin	<0·09	<0·07
Coumarin	1·0	1·0	5:7-dihydroxycoumarin	<0·20	<0·40
3-methylcoumarin	0·56	0·66	6-methoxy-7-hydroxy-coumarin	<0·10	<0·40
Melilotic lactone (3:4-dihydrocoumarin)	0·32	0·23	5:7-dihydroxy-4-methylcoumarin	<0·09	<0·07
6-chlorocoumarin	0·31	0·57	4-methyl-5:7-di-methoxycoumarin	<0·16	<1·01
3-chlorocoumarin	0·25	0·62	4-methyl-5-acetoxy-7-methoxycoumarin	<0·12	0·43
Coumarin-3-carboxylic acid	0·15	0·27	4-methyl-5-hydroxy-7-methoxycoumarin	<0·10	<0·51
3-hydroxycoumarin	0·18	0·45	4-methyl-5-methoxy-7-hydroxycoumarin	<0·10	0·24
4-hydroxycoumarin	0·13	0·40	5:7-dihydroxy-3:4-di-methylcoumarin	<0·10	—
6-hydroxycoumarin	0·10	0·07	4-methyl-7-hydroxy-coumarin	<0·09	—
7-hydroxycoumarin	0·09	0·20			
Coumarin-3-carboxylic acid ethyl ester	0·03	0·71			
6-nitrocoumarin	<0·10	0·27			
Tribromocoumarin	≪0·48	≪2·0			
Tetrachlorocoumarin	<0·28	1·23			
7:8-dihydroxycoumarin	<0·09	<0·07			

TABLE II

Inhibition indices of compounds related to those in Table I

Compound	Inhibition Index	
	Lettuce	Wheat
Resorcinol (1:3-dihydroxybenzene)	0·11	—
Catechol (1:2-dihydroxybenzene)	0·05	—
Pyrogallol (1:2:3-trihydroxybenzene)	0·05	—
Carbostyril (2-hydroxyquinoline)	0·05	0·18
Dicoumarol	0·08	—
2-methyl-5:7-dihydroxychromone	0·10	0·37
2:4-dichlorophenoxyacetic acid	5·0	0·66
2:4:6-trichlorophenoxyacetic acid	1·1	1·55

A number of observations on some other compounds listed in Table II may be mentioned here, although they do not directly contribute to the central problem. The phenols decrease in activity as the number of hydroxyl groups increase: resorcinol>catechol>pyrogallol>gallic acid. It is also worth pointing out that dicoumarol, a known anti-blood-coagulant, is quite inactive as a germination inhibitor.

(*b*) *Wheat.* The general picture is entirely analogous to that of lettuce and need not therefore be enumerated in detail. Substitution in the coumarin system is, however, not quite as effective in destroying the activity as an inhibitor as in the case of lettuce.

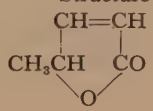
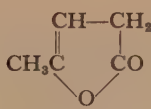
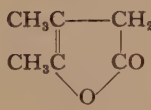
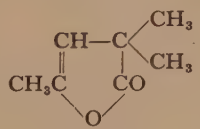
One notable exception is of particular interest, tetrachlorocoumarin is more active than coumarin as a germination inhibitor for wheat. It is therefore the only instance met with in this investigation where a derivative of coumarin surpasses coumarin itself in activity. It is also of interest to note that this is only the case for wheat and not for lettuce.

2. *Pentacyclic compounds*

The activity of some pentacyclic lactones are listed in Table III. It will be seen that 2-pentene-1:4-olid, which contains an unsaturated lactone structure, has an activity about half that of coumarin. All the other pentacyclic compounds have a far lesser activity than coumarin. Apparently the unsaturated lactone structure with the double bond one carbon removed from the —CO group is here also of great importance.

TABLE III

Inhibition indices of some 5-atom-ring compounds

Compound	Structure	Inhibition Index	
		Lettuce	Wheat
2-pentene-1:4-olid		0·42	0·50
3-pentene-1:4-olid		0·08	0·33
$\Delta^{\beta\gamma}$:β-methylangelicolactone		0·105	0·40
$\Delta^{\beta\gamma}$:αα-dimethylangelicolactone		0·12	0·19
α-oxycrotonolactone		0·7	—

The position in lettuce and wheat is analogous, but the differences between the activity of the various lactones are smaller, and the importance of the unsaturated structure is less marked in the case of wheat.

DISCUSSION

Much of the data obtained in these experiments is negative in nature in that they only eliminate certain possibilities regarding the possible action of coumarin.

Any form of substitution in the coumarin molecule partially destroys its activity, with the single exception of tetrachlorocoumarin. Substituents of all the known activation and deactivation effects on the benzene nucleus were tested ($+I+T$, hydroxyl group; $-I-T$, nitro group; $-I+T$, chloro group where $-I>+T$ and the alkoxyl group where $+T>-I$).[1] The resultant electron shift and redistribution in no case caused increased activity. The cause for the activity of coumarin must therefore be sought in its specific structure and the activity of certain positions in the molecule.

The structural features which are determining are: the presence of the double bond in the lactone ring and the occurrence of the lactone structure. Removal of the double bond at once reduces activity (cf. coumarin and melilotic lactone, Table I). Destruction of the lactone structure has a similar effect, e.g. the replacement of —O— by —NH— (cf. coumarin, Table I, with carbostyril, Table II). The importance of the lactone is also confirmed by the activity of 2-pentene-1:4-olid which is an unsaturated lactone and the low activity of 3-pentene-1:4-olid which is not.

It is remarkable that the halogenated coumarins have only one- to two-thirds the activity of coumarin itself, as was suggested by Audus and Quastel (1947). This is particularly so for lettuce, while in wheat halogen substitution does not as readily abolish activity. In the case of tetrachlorocoumarin in wheat there is an increase of activity as compared with coumarin.

Lettuce and wheat seem to differ in their sensitivity towards halogen-substituted coumarins.

Coumarin derivatives behave alike as germination and root-growth inhibitors. Some differences in their activity as germination inhibitors and as root-growth inhibitors as described by Goodwin and Taves (1950) must, however, be recalled. 7:8-dihydroxycoumarin, coumarin-3-carboxylic acid, and scopoletin are almost inactive as germination inhibitors (Table I), while they were as active as, or more active than, coumarin as root-growth inhibitors. The reverse is the case for 3-methyl substitution which greatly affected root growth but not germination inhibition.

The role of the aromatic nucleus as a determining factor in coumarin activity was not established as we were unable to study hexacyclic lactones. The observations of Kuhn, Jerschel, Moewus, and Möller (1943) that parasorbic acid is less active than coumarin and of Veldstra that α-pyrones are inactive while 6-phenyl-α-pyrone approaches coumarin in activity point to the importance of the aromatic nucleus. This is also indicated by the low activity of pentacyclic lactones.

[1] I is the inductive and T the tautomeric effect, + indicating activation and — deactivation of the aromatic ring.

The lack of activity of coumarin derivatives cannot be sought in their failure to enter the seed or embryo. If this were the case there should be a marked difference in activity in hydroxycoumarins and esterified hydroxycoumarins due to differences in their permeability. In fact both are inactive.

The action of coumarin must be sought in its specific action on one or more enzyme systems operating during germination. Thimann and Bonner (1949) suggest that coumarin acts on enzyme containing the —SH group by addition of the enzymes to the 3:4-position of coumarin. This is not borne out by the observation (Mayer and Evenari, 1951) that cysteine enhances the inhibitory action of coumarin and does not compete with it, nor by the data of Cavallito and Haskell (1945) that coumarin is one of the unsaturated lactones not reacting with cysteine. The blocking of the 3-position by methyl substitution is not particularly effective in reducing activity, while substitution in the 6- or 7-position reduced activity very markedly. This confirms the view of Goodwin and Taves that coumarin activity cannot be ascribed to the reactivity of the double bond in the 3:4-position with —SH groups.

The results here quoted do not lend support to the view that coumarin acts by reacting with —SH containing enzymes, at any rate when acting on the germinating seed.

It is as yet too early to speculate what specific part or parts of metabolism are affected. This part of the problem is now being approached by the study of the effect of known metabolites and inhibitors of various stages of metabolism on the inhibition obtained by coumarin and 2:4-D. From this work it has already emerged that coumarin and 2:4-D affect different parts of metabolism of the germinating seed.

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The Influence of Mature Tissue on Division in the Meristem of the Root

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SUMMARY

Observations have been made on rates of increase in total number of cells and on rates of division in isolated pea roots grown in sterile conditions in one series over a period of 9 days and in another started from tips of different lengths. It is shown that when cultures are started from tips of 6·0 mm. excised from germinated seeds the rate of increase in cell numbers increases with time until the fifth day and then decreases to the ninth day. It is further shown that when cultures are started from tips of 3·0, 6·0, and 10·0 mm., the initial lag phase is longer, the peak rate in division occurs later, and the peak value tends to be greater the shorter the initial tip. These observations are taken to indicate that division in the meristem depends partly on the synthesis of appropriate metabolites in the meristem and partly on a supply of metabolites from mature regions of the root.

INTRODUCTION

THE present investigation was designed as a contribution to an elucidation of the conditions that control division in the meristem of the root, the particular phase of the situation that has been analysed here being the effect of mature tissues on the activity of the meristem. In a root that is growing normally attached to a shoot there is undoubtedly a flow of nutrients along it from which the meristem is supplied. The nature of these nutrients, however, is a matter of some doubt. It is frequently assumed that the meristem has the same metabolic pattern as other active cells in the plant and that (when it is supplied with certain micronutrients) it can synthesize all the metabolites it requires from simple carbohydrates and inorganic ions. For such a view there is no unequivocal evidence and there are some indications to the contrary.

The development of the sterile root-culture technique by Robbins (1922) and by White (1932, 1933, 1934) provides an opportunity for an examination of this problem. The detached root grows immersed in a solution whose composition is known and which is immediately available to all parts of the growing system. It may be absorbed as readily by the meristem as it is by the mature regions of the root. In this situation, if the meristam can synthesize from the primary nutrients in the medium all the metabolites required in division, then activity in it should be independent of the mature regions of the root. Two approaches to this situation immediately suggest themselves: an analysis of meristematic activity during the course of growth, and the induction of growth with different quantities of mature tissue attached to the meristem. With the first, if meristematic activity is independent of activity in mature tissue, then the extent of division should not change as growth

proceeds and the mass of mature tissue increases. With the second, if the two parts are independent, then the mass of mature tissue in the initial inoculum should not affect the situation.

In systems such as cultured roots, continued growth undoubtedly depends primarily on division in the apex, and whatever index of growth is taken it must in some sense reflect conditions in the meristem. Hitherto the growth of isolated roots has been defined primarily in terms of growth in length. This standard of reference is a convenient one and its application has yielded valuable results. The length increment, however, is a complex function which depends not only on division but also on cell enlargement. The use of it as an index of meristematic activity is further complicated by the fact that in the course of growth the morphology of the root probably changes in such a way that the number of cells across the root decreases. If this happens then the contribution of a unit number of cells of the same mature length and volume to the total length increment will vary. Clearly for an analysis of meristematic activity an estimate not of length but of the total number of cells is required. This has been made here by an application of the technique originally developed by Brown and Rickless (1949). Length, however, has been measured as a routine procedure, and length data are given below for comparison with the results of earlier workers.

The experimental material of Brown and Rickless was inadequate for this investigation since the growth made with it is limited. We have used pea roots. These were chosen since other observations were being made with them in this laboratory and the conditions for culturing them had already been investigated by Bonner and Addicott (1937). In the event it was found that pea roots provide particularly suitable material for the analysis of the relation between activities in mature and meristematic regions since the nutrient reserve carried in the meristem is apparently small.

MATERIALS AND METHODS

The experimental design is a simple one. For each experimental treatment a number of roots are cultured and from this number samples of several roots are withdrawn at intervals of 24 hours. The length of each root, the total number of cells, and the number of meristematic cells in the sample are determined. From these measurements an average length and average number of total and meristematic cells are obtained. From the average total and meristematic numbers of cells a rate of division is calculated. Thus the final result is a series of average lengths, average total numbers of cells, and rates of division in terms of which the growth may be analysed.

The initial inocula for the cultures are obtained from seeds germinated in sterile conditions. After treatment with alcohol and 0·1 per cent. mercuric chloride, seeds are washed in sterile distilled water and transferred to a Petri dish in which they are incubated with sterile water for 3 days at 25° C. At the end of this period roots have emerged and the tips are excised with a sterile

scalpel, the lengths being adjusted by cutting against a scale marked on a sheet of filter-paper placed below the dish. The tips are transferred to 15 ml. of liquid medium in a Petri dish, two tips being allocated to each dish. The cultures are incubated at 25° C. usually for 7 days. The present series of observations are all based on cultures from such root-tips and subcultures from these have not been used in this phase of the investigation.

The basic medium that has been used throughout consists of an inorganic salt mixture dissolved in 4 per cent. sucrose. The medium is that recommended by Bonner and Addicott (1937) for pea roots. For the material of this investigation it has been used without any vitamin supplement. All solutions have been sterilized by autoclaving.

For each treatment in any experimental series about 50 dishes are set up. If chance contamination is only sporadic this number provides 7 samples of 5 dishes each. Occasionally the frequency of infection is such that the number of dishes in each sample has to be reduced or the length of the experimental period curtailed. In the majority of experiments, however, samples of 10 roots are taken on each day over a period of 7 to 9 days.

On each sampling occasion 5 dishes are removed at random. The length of each root is measured against a ruler. The 10 roots are then cut into lengths of about 1 cm. and the fragments immersed in a known volume of 5 per cent. chromic acid. After treatment in the acid for 24 hours the suspension is repeatedly drawn into and expelled under pressure from a pipette with a fine jet. The numbers of all cells and of meristematic cells in the suspension are then determined by the application of a haemocytometer technique.

From these results an average total number of cells and an average number of meristematic cells in the root may be calculated. If during any interval the whole increment in cell number is due to the activity of the apical meristem, and if all the so-called meristematic cells are all in the apical meristem, then a figure for the rate of cell division can be calculated from these two sets of data. The increase in the total number of cells during any interval divided by the average number of meristematic cells for that interval gives the number of divisions per meristematic cell per interval (in this case, 24 hours). Thus the rate is derived from the expression $(N_2-N_1)/\{\frac{1}{2}(M_1+M_2)\}$ where N_1 and N_2 are the total number of cells and M_1 and M_2 the numbers of meristematic cells at the beginning and end of the interval respectively. It may be emphasized that the rate is only a relative figure. In this investigation cells have been counted as meristematic when they do not show a prominent central vacuole and when they are approximately isodiametric. The standard is based on the examination of squash preparations and all the cells covered by it are probably meristematic, although it probably does not embrace some cells that may divide. At the same time since there is some evidence (Brown, 1951) that the physiological differentiation of the meristem is constant whatever its size, the number of so-called meristematic cells probably bears some relation to the number of potentially dividing cells, and therefore probably provides a relative figure of some significance particularly when all the counts are made by the

same observer. It has been found in this investigation that counts of 'meristematic' cells by different observers may differ by 20–30 per cent.

As indicated above in investigations such as this the rate of division can only be calculated from the gross data when it can be assumed that the total increment is due to the activity of the apical meristem and when it can be assumed that all the meristematic cells are in the terminal meristem. Clearly these conditions are not fulfilled if secondary thickening or extensive lateral root formation are involved. In the present series of cultures, as in those of Torrey (1951), secondary thickening has not occurred. This has been shown by direct anatomical observation and by determining the number of cells in a mature region of the root over a period of 24 hours. Lateral root formation, on the other hand, does occur. In this connexion two aspects of the situation must be distinguished, the production of primordia, and the development of these into lateral roots. The data of Table I show that in a typical series of cultures on the seventh day there is on the average less than one lateral per root. Thus the number of expanded cells in the whole culture arising from lateral roots is negligible. On the other hand, the number of primordia on the seventh day is substantial (9·1). These contain only meristematic cells and if the number of them is large it might affect the significance of the calculated value for the rate of division. An estimate of the number involved may be made by determining the number of meristematic cells in the terminal centimetre and in the rest of the root. The results of such an estimation are shown in Table I. It is evident from these data that no lateral root primordia occur until the fifth day and that the number of meristematic cells involved is negligible until the seventh day. Even on the seventh day, however, since the number of apical cells affects both the numerator and the denominator of the fraction from which the rate is calculated, it does not affect significantly the magnitude of the calculated value. When the extra apical meristematic cells have been separately determined, the number of these can be deducted from the total and the gross number of meristematic cells, giving a figure from which more accurate values for the rate of division can be calculated. Such corrected values are given in brackets along with the gross values in Table I. It is evident that until the seventh day the two values do not differ greatly. Since the separate estimation of extra apical meristematic cells does not change significantly the value of the rate of division, this elaboration was not incorporated in the second series of experiments described below.

In this paper the term meristematic cell is used with the qualifications indicated above, and the term vacuolated cell in the sense of one that has developed a well-defined central vacuole which is immediately apparent after the tissue has been macerated in chromic acid.

RESULTS

Two groups of results are presented in this section. The first provides a detailed analysis of the growth of roots over a period of 9 days; and the second

shows the effect of the length of the initial tip on the subsequent growth of the roots.

I. *Changes in the course of growth*

The results of one experiment which was continued for 9 days are shown in Table I. Some of the results of another experimental series in which the induction of lateral root primordia was being studied are also included in this table (columns *P* and *LR*). In this experiment the cultures were started from 6.0-mm. tips excised from germinated seeds.

TABLE I

Growth of roots cultured from 6·0-mm. seed tips

T, time, days; *L*, length, mm.; *N*, total number of cells, thousands; M_1, number of meristematic cells in apex, M_2, in lateral root primordia, thousands; *RD*, number of divisions per meristematic cell per 24 hours; *P*, number of lateral root primordia; *LR*, number of emerged lateral roots.

T	*L*	*N*	M_1	M_2	*RD*	*P*	*LR*
0	6	275	140	—	—	—	—
1	10	290	100	0	0·13	0	0
2	15	335	90	0	0·47	0	0
3	23	390	80	0	0·65	0	0
4	35	450	55	0	0·86	0	0
5	47	530	56	4	1·43 (1·36)	—	0
6	59	600	54	16	1·27 (1·10)	6·8	0
7	70	655	55	20	1·00 (0·92)	9·1	0·7
8	79	700	50	30	0·90 (0·70)	10·5	0·8
9	87	740	55	35	0·75 (0·55)	12·3	1·8

The values for length and total number of cells in Table I are reproduced in Fig. 1. Over the whole 9 days length increases approximately at the rate of about 1·0 cm. per day, which compares favourably with the results obtained by Bonner and Addicott (1937) for the same material.

It is evident from the curves of Fig. 1 that the length increment is closely dependent on the rate of increase in the total number of cells. With both, the rate of increase is low at first and tends to increase with time. It may be noted that the length increase is not uniquely determined by number increase at all stages. During the first 24 hours an increment in length of 4·0 mm. is accompanied by an increase of 15,000 in the number of cells. During the second 24 hours, however, an increment of 5·0 mm. is associated with an increase of 45,000 cells. Clearly while growth in length from 24 to 48 hours is being determined partly by cell division, during the first 24 hours it is not. The counts of meristematic cells indicate that during the first 24 hours increase in length is being determined primarily by the vacuolation of meristematic cells carried over from the tip of the parent seed root. This phenomenon is strikingly shown by certain of the data of Table II. In cultures from 3·0-mm. tips although there is no increase in the number of cells during the first 3 days the length of the root increases from 3·0 to 18 mm. During the same period the

number of meristematic cells decreases from 120,000 to 60,000. Evidently these meristematic cells carried over from the parent root vacuolate, and by so doing promote the increase in length of the cultured root.

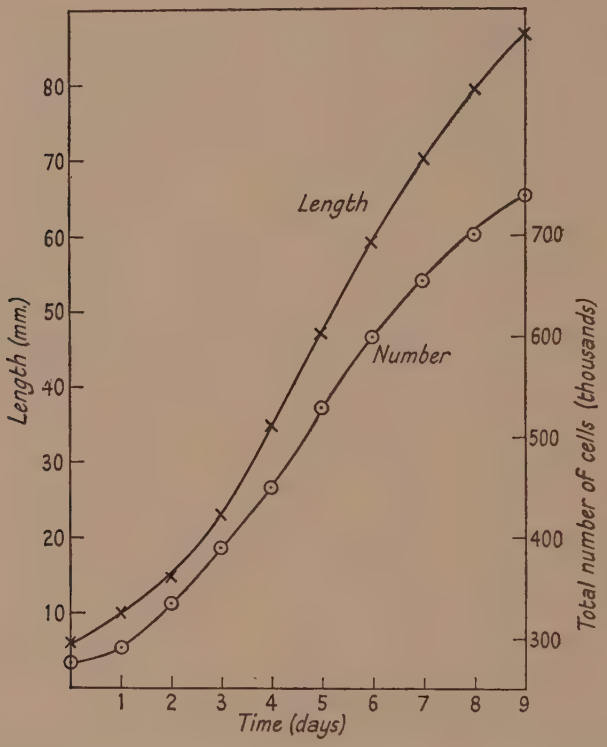

FIG. 1. Growth of roots from 6·0-mm. tips, showing length and total number of cells

It is evident from Tables I and II that the meristem is a variable region. The data suggest that after some cells are formed in the extreme tip, changes occur in them which lead to vacuolation and subsequent expansion. When cell division ceases the changes leading to vacuolation proceed with the result that the meristem shrinks. When division is active, on the other hand, cells after formation may divide again and in any case the cells that vacuolate are replaced by others from the extreme tip with the result that the size of the meristem tends to be larger.

As pointed out above, when growth is vigorous length increase over the whole experimental period is related primarily to the increase in the number of cells. It is significant, however, that the rate of increase in the number of cells changes with time. The curve showing the increase in the number of cells with time is S-shaped, and indicates the incidence of two phases. During the first the rate is increasing and during the second it is decreasing.

This succession has been observed repeatedly in a variety of cultural conditions, and the following possibilities have been considered:

(1) that the early lag is due either to an injury effect, or
(2) to a relatively slow absorption from the medium by the meristem;
(3) the second phase may be the result of the induction of primordia, or
(4) to the exhaustion of some essential metabolite in the tip;
(5) the cycle of changes may be the result of changes in the medium induced by the root itself, or
(6) of an interaction of metabolites synthesized in mature and meristematic regions.

It is probable that the first five possibilities can be disregarded. Brown and Rickless (1949) have shown with *Cucurbita* that when roots are cut as near as 1·5 mm. to the tip vigorous division can nevertheless still proceed in the excised fragment, and the continuation of growth in the cells near the cut surface indicates that the activity of these is not inhibited by injury effects. Data are not available on relative rates of absorption in different parts of the cultured root, and the possibility must be considered that absorption from the medium is relatively slow in the meristem, and that normally the nutrient requirements of the meristem are met by absorption through mature tissue followed by transport to the meristam. If this is the case, then as the mass of mature tissue increases, the supply of nutrients from the medium to the meristem is likely to increase. This possibility has been examined by culturing tips in media with double the normal concentration of nutrients. This treatment, however, far from accelerating division actually depresses it. Table I shows that at the stage at which division in the meristem is decreasing primordia are being formed and it is possible that the development of the latter leads to an inhibition of the former. On the other hand, when the initial tip is a long one (Fig. 2) there is little or no change in the rate of cell increase although primordia are formed with these as with other cultures. There is some evidence available that at 7 days the tip is vitamin deficient. Subcultures have shown that when 5·0-mm. tips are taken from cultures such as those of Table I, they respond immediately to vitamin supplement. On the other hand, 20-mm. tips do not (Wightman and Brown, 1951). Evidently on the seventh day the tip is deficient in vitamins, but the tissue 20 mm. from it is not and this supplies the tip with sufficient vitamins or with substances formed from them. The evidence of Fig. 2 also suggests that the exhaustion of a metabolite reserve is not the cause of the decrease in the rate of division. Although with a 10-mm. tip about the same number of cells are formed (300,000) during 7 days as with the 6·0-mm. tip, nevertheless with the longer tip there is no indication of a decreasing rate of cell increase after the fifth day.

The possibility that the succession of phases is due to changes in the medium has been examined by introducing tip inocula into media in which roots have already been cultured, and transferring roots on the fourth day to fresh media. It was found that neither of these treatments had any significant effect on the rate of cell increase.

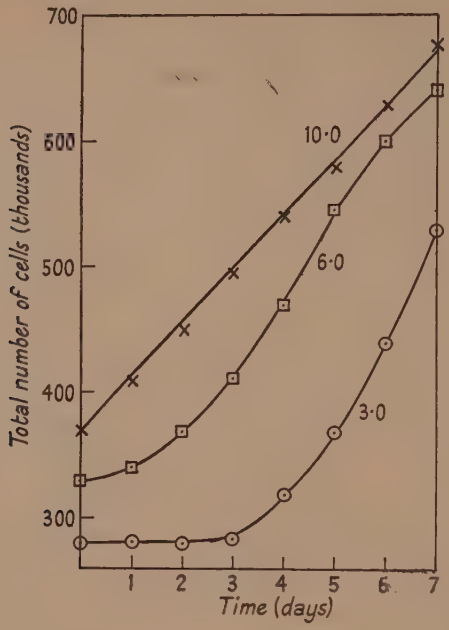

FIG. 2. Increase in total number of cells in roots from 3·0-, 6·0-, and 10·0-mm. tips

The final possibility remains that the succession of phases is due to an interaction of materials synthesized in the tip and in the mature region of the root. It is significant that changes in the rates of cell increase are accompanied by corresponding changes in the rate of division. Clearly changes in the former are being determined by the latter, and the data suggest that in the early phase the rate of division increases with increase in length of the root. Division, it may be suggested, depends partly on substances generated in mature regions of the root and that as this increases with increase in the mass of mature tissue it stimulates an enhanced rate of division. Evidence presented below supports this interpretation and indicates that the final decrease is due to an interaction between rate of production of substances in the meristem and in mature regions.

II. *Effect of length of initial tip on growth*

If the early acceleration with the 6·0-mm. tip shown in Table I is an effect of increasing supply of mature product, then the early lag should be more pronounced with shorter tips and less pronounced with longer ones. The results of one experiment designed to test this possibility is shown in Table II. In this experiment the effects on subsequent growth of establishing cultures from 3·0-, 6·0-, and 10·0-mm. tips are compared.

TABLE II

Growth of roots from 3·0-, 6·0-, and 10·0-mm. tips

M is total number of meristematic cells (thousands); rest of symbols as in Table I.

	3·0				6·0				10·0			
T	*L*	*N*	*M*	*RD*	*L*	*N*	*M*	*RD*	*L*	*N*	*M*	*RD*
0	3·0	280	170	—	6·0	330	170	—	10·0	370	160	—
1	7·5	282	110	0	9·0	340	125	0·07	17·0	410	105	0·30
2	11·0	280	86	0	14·0	370	95	0·27	22·0	450	80	0·44
3	18·0	284	60	0·06	22·0	410	90	0·48	28·0	495	70	0·60
4	22·0	320	50	0·65	31·0	470	70	0·75	35·0	540	65	0·67
5	30·0	370	53	1·00	41·0	545	65	1·12	44·0	580	68	0·60
6	44·5	440	52	1·40	52·0	600	60	0·90	54·0	630	70	0·73
7	62·0	530	57	1·63	69·0	640	60	0·66	73·0	675	64	0·67

The total cell number data of Table II are reproduced in Fig. 2. It is evident from these data that mature tissue controls the activity of the meristem very intimately. With the 10·0-mm. tip the rate of increase in cell numbers is more or less constant throughout the experimental period and so also is the rate of division. With the 3·0-mm. tip, on the other hand, there is a lag period of at least 3 days during which there is no increase in cell numbers, and thereafter the rates of cell increase and of division increase with time. The cultures from 6·0-mm. tips are intermediate between these two extremes. The 3·0- and the 10·0-mm. tip carry the same number of meristematic cells. Whereas the 10·0-mm. tip has 210,000 vacuolated cells, the 3·0-mm. tip only has 110,000. Thus it is probable that division in the 10·0-mm. tip can proceed immediately by reason of the large number of mature cells that it contains. It is probable that division depends at least partly on a supply of products from the mature region of the root and that in the 10·0-mm. tip there is a sufficient mass of mature tissue to meet the immediate needs of the meristem. In the 3·0-mm. tip, on the other hand, not only are there a smaller number of fully vacuolated cells but it is probable that there are few fully mature cells. Thus no cells are available from which the product from mature tissue can be supplied. As a result there is a lag period of 3 days during which cells are vacuolating from the meristem and maturing. Division begins on the third day, and it is significant that it is not until this time that the root from the 3·0-mm. tip contains the same number of vacuolated cells (224,000) as the original 10·0-mm. tip (210,000). On the other hand, while there is a long lag phase with the 3·0-mm. tip, the rate of division eventually reaches a higher value than it does with the 10·0-mm. tip. It reaches a value of 1·63 on the seventh day, whereas with the 10·0-mm. tip the rate of division remains constant at approximately 0·70. This difference suggests that division depends on two sets of metabolic products, one provided from mature tissue and a second formed in the meristem. With the 3·0-mm. tip there is no supply of mature product during the first 3 days, but during this time material that is formed in the meristem is accumulating. Thus as the supply of mature product increases it promotes an enhanced rate of division

which continues to increase until the accumulated reserve of tip product is exhausted. In the 10·0-mm. tip there is no lag in the production of mature product and tip product therefore does not accumulate. In this series the rate of division is limited by the rate of production of tip material which remains constant. The 6·0-mm. tip contains the same number of meristematic cells as the other two but carries an intermediate number of vacuolated cells (160,000). It is significant, however, that the characteristic growth it promotes is intermediate between those induced by the shorter and the longer tips. With the 6·0-mm. tip the lag phase is shorter and less intense, and the rate of division increases more rapidly than it does with the 3·0-mm. inoculum. With the 6·0- as with the 3·0-mm. tip the rate of division increases until a peak value is reached, but in this case the peak is reached earlier and thereafter the rate decreases. Moreover, the peak value is lower than the maximum value recorded with the 3·0-mm. tip. Since in the 6·0-mm. tip the mass of mature tissue is greater than it is in the shorter fragment, the production of mature product increases more rapidly, with the result that it promotes a more rapid increase in the rate of division. Further, the more rapid production of mature product involves a smaller accumulation of tip product with a consequent lower and earlier peak. As the reserve is exhausted the rate falls and reaches the value characteristic of the 10·0-mm. series which is determined by the rate of production of tip product.

DISCUSSION

The nature of the product that is synthesized in mature tissue and supplied to the meristem is unknown. Attempts have been made to break the lag phase by supplying a variety of substances to short tips, but so far without success. The possibility cannot be disregarded that the influence of mature tissue is due to a substance of the leptohormone type (Haberlandt, 1922). It is possible that as the quantity of phloem material increases, so also does the supply of a particular hormone generated in it which promotes division in the meristem. On the other hand, in the experiments of Haberlandt the effect of the leptohormone was demonstrated with mature cells, and it is probable that the requirements of mature and meristematic cells with respect to division are different. Further it has been shown (Robinson and Brown, 1952) that the metabolic pattern of meristematic cells differs considerably from that of mature cells. The data suggest that each may contain enzyme systems which are either absent or are only poorly developed in the other. Thus the suggestion that mature tissue produces a variety of metabolites which are required by the meristem but which cannot be synthesized in it is compatible with the evidence presented above and with evidence assembled in other investigations.

Whatever the nature of the mature product, however, it is clear that when observations are being made on division in the meristem of an intact root the differences observed with different treatments may not be due to immediate effects on the meristem itself.

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ERRATUM

'A comparison of the aerobic and anaerobic respiration of apples' by J. C. FIDLER. *J. exp. Botany*, II, 41–64.

p. 63, equation (4) should read:

$$\left.\begin{array}{l}\text{Pyruvic acid} \rightarrow \text{Acetaldehyde} + CO_2 \\ \text{and, Acetaldehyde } (+H_2) + O_2 \rightarrow CO_2 + H_2O\end{array}\right\} \quad \ldots\ldots\ldots \quad (4)$$

An Electron Microscope Study of the Spermatozoid of *Sphagnum*

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WITH PLATES I—X

SUMMARY

The micro-anatomy of the cilia of *Sphagnum* has been elucidated in greater detail than in the ciliary types previously investigated. The facts are summarized in a diagrammatic reconstruction.

THE spermatozoids of *Sphagnum* used in this study were obtained on two different occasions separated by an interval of 2 years. Both originated as class material for undergraduate courses on Bryophyta and both can be referred taxonomically to the *S. acutifolium* aggregate, although they are probably not the same species since the spermatozoids of the first sample are smaller than those of the second and they differ also in the relative dimensions of the body and cilia. In other respects they are in very close agreement, and it does not seem necessary therefore to explore the minutiae of their taxonomic position more exactly, though as a precaution we have retained some dried material of both species, which can be consulted by anyone interested. For the present purpose, however, it seems sufficient to designate the first species, which was sent in from Austwick, Lancashire, in November 1949, as *S. acutifolium* agg. species 1. It is represented by the two ultraviolet photographs included in Plate I. The second species, which came in from Aberystwyth, Wales, in the autumn of 1951, can then be designated *S. acutifolium* agg. sp. 2. It has supplied Fig. 3, Plate I, and the electron micrographs reproduced in all the remaining plates.

On both occasions the spermatozoids were obtained by dissecting out male branches in a drop of water on a slide or in a watch-glass. The antheridia are easy to find and they dehisce readily. The spermatozoids do not, however, always swim readily after they have emerged, but are liable to remain permanently coiled up and motionless. This difficulty was very pronounced in 1949, and much patience was needed to obtain enough motile cells for the preliminary study with the ultraviolet microscope. Swimming was fortunately more vigorous in 1951, and to this fact can be attributed some at least of the exceptional clarity of the preparations. These were made by pipetting off the swimming spermatozoids from the slides on which they had been liberated and redepositing them on prepared formvar films. Killing was by 30 sec. exposure to the vapour of 2 per cent. osmic acid in the usual way and after

drying, washing in distilled water, and again drying, they were shadowcas with gold palladium. The other technical details and the instruments used ar exactly as in our previous papers on plant cilia. All the electron micrograph on this occasion were taken at 60 kV. and, as in previous papers, the magnifica tions quoted should be regarded as approximately correct but not exact.

GROSS MORPHOLOGY OF THE SPERMATOZOID

When first liberated from the antheridium each spermatozoid is closel coiled within a transparent vesicle. With visual light the most conspicuou organ is a small plastid, and the nucleus, cilia, and residual protoplasm are s transparent that they are difficult to see. With ultraviolet light the plastid though still conspicuous, has less light-stopping power at the wave-lengt used (2,750 Å) than has the nucleus which, in consequence, is more readil located (Plate I, Fig. 1). With the electron microscope, nucleus, cilia, an plastid are almost completely opaque and appear black (Plate II, Fig. 3).

As the enveloping membrane disintegrates it develops a fine fibrillar mark ing (cf. the centre of Plate IV, Fig. 10), and shreds of this can often be detecte in the field of the electron microscope after the cell has emerged. Examples o this are visible on the right of the body in Plate V, Fig. 11, or spread over th ground at the left-hand end of Plate II, Fig. 4, or as shreds lying across th intact cilium in the lower part of Plate III, Fig. 9. In some other Bryophyt it is possible to pick up empty skins, with no visible internal structure, afte the spermatozoids have finally emerged, but this is not the case in *Sphagnum*

On disintegration of the skin the cilia are commonly the first organs t emerge, and they may become partially motile even before they are full uncoiled, as in the upper cell of Plate I, Fig. 2; the 'head' comes out nex and the body last. The plastid may adhere to the body for a short time an travel with it, but it is commonly lost and plays no further part. The bod itself is a very beautiful object in the living condition and has a shape whic is not represented by any of the illustrations reproduced here. The watch spring attitude of dormancy is replaced, on emergence, by that of a solenoi with the gyres widely spaced, and the whole cell rotates as it swims in a manne which is well known. Unfortunately the act of drying, which is essential fo electron microscopy, destroys the natural attitude of the body completely and the straightness of the back end of the body in the two pictures displayin it (Plates II and V, Figs. 4 and 11) is an entirely unnatural attitude as is th somewhat similar posture of the lower cell of Plate I, Fig. 2.

With regard to the cilia, their slightly unequal length and their attachmen at two separate points on the front end of the cell are well-known facts, whic are displayed to advantage in Plate I, Figs. 1 and 2. In the intact state thei distal extremities taper to a point which is narrower and more transparen to both ultraviolet light and the electron beam, as may be seen in Plates and III, Figs. 1, 2, 8, and elsewhere. These apices are, however, peculiarl vulnerable to post-mortem changes, some of which are very instructive (c p. 270 below), and their appearance may then alter considerably.

At the base, each cilium is attached to and, in the case of the front flagellum at least, embedded in (cf. Plate III, Fig. 9) a translucent gelatinous structure, the 'head'. This organ can be seen in both the ultraviolet photographs (Plate I, Figs. 1 and 2) and in the electron micrographs of Plates II, III, and V, Figs. 4, 9, and 12. In both Plates III and V, Figs. 9 and 12, the material of which the 'head' is composed appears to have split near the front end to reveal the pointed tip of the nucleus within, but there is no trace of any special organ of attachment at the base of each cilium comparable to the 'bulbous base' in the fungi (Manton, Clarke, Greenwood, and Flint, 1952) or the 'basal granule' of *Fucus* (Manton and Clarke, 1951*c*).

The nucleus itself, where it forms the 'body' of the spermatozoid, appears to be covered by a skin which has generally split as the body has straightened (cf. Plates II and V, Figs. 4 and 11). It is probable that this skin is of cytoplasmic origin and does not correspond to a nuclear membrane in the usual sense of the word. It commonly ends in a curious little appendage of five fine fibrils (cf. Plate II, Fig. 5), though these may sometimes be invisible (Plate V, Fig. 11) or be reduced by breakage. What significance, if any, should be attached to this tailpiece is uncertain. It may be suspected of having no functional significance other than as the remains of a former attachment of the body to some other part of the original cell, such as the plastid, which has fallen away. Whether or not any residual protoplasm is normally present outside the skin is uncertain. The ultraviolet photograph of Plate I, Fig. 2 suggests that there is some on the outside of the body but it is not detectable in the electron micrographs once the cell has fully emerged. Some allowance must, however, perhaps be made for artificial removal of material of this kind in the process of washing as the preparations were being made.

FIBRILLAR COMPOSITION OF THE CILIA

The most conspicuous thing about all the electron micrographs except Plates I and III, Figs. 3 and 8, is the extent and completeness of fibrillar disintegration of the cilia. In certain cases, e.g. Plates II and III, Figs. 6 and 9, one cilium is dismembered and the other intact, a difference which is generally due to a different degree of emergence from the vesicle. In other cases both cilia are dismembered, though one is more easily analysed by the eye than the other, e.g. Plates II and V, Figs. 4 and 11. In yet other cases, e.g. Plate IV, Fig. 10, both cilia are completely dismembered and equally suitable for numerical analysis.

Among the figures reproduced, which are only a selection from many other analysable specimens, complete numerical analysis is possible in Plates II and V, Figs. 4, 6, 12, and 13, each on one cilium only, but both can be used in the cell of Plate IV, Fig. 10, although owing to the shape of the space available not all of both cilia can be accommodated in the plate. Portions of other cilia which have been included for other reasons but in which the numerical analysis can equally well be made are in Plates VIII and X, Figs. 20 and 23.

There is no variation in any of these specimens and in many others not included in the plates. Whenever dismemberment is complete, the number of strands is exactly that to which we have now become accustomed in other groups, namely, eleven strands, two of which are different from the others and centrally placed. The two central strands are slightly unequal in length in this material and they adhere more closely together than do the others: their duality can, however, generally be clearly seen at the tip; cf. Plate II, Fig. 7.

This closer cohesion of the central strands can now be stated to be due to the presence of a skin surrounding the two strands although not extending quite to their tips. This can be seen in Plate II, Fig. 7, which is an enlarged view of the termination of the central strands of Plate II, Fig. 6. At the extreme end the two component strands, of slightly unequal length, can be clearly distinguished lying side by side. In this region the shadow (appearing white in the direct print) is narrow, indicating that the strands themselves have not very much thickness above the surface of the formvar. As the eye passes back from the apex of the strands the cleft between them is abruptly obscured by a layer of superposed material and simultaneously the shadow becomes wider, indicating that the pair of strands, when enclosed by the sheath, project more from the formvar surface than do the naked strands alone. All this indicates unmistakably that the reason why these strands separate less readily than the others elsewhere in the cilium is that until the sheath disintegrates they are held together by it.

ARRANGEMENT AND MODE OF COHESION OF THE PERIPHERAL STRANDS

That the nine peripheral strands are arranged in the form of a tube surrounding the central pair is clearly indicated by a specimen such as that of Plate VIII, Fig. 19, in which part of the central pair, still in its sheath, has remained in position. That the peripheral strands forming the surrounding tube have a slightly spiral path along the length of the cilium is further indicated by a configuration such as that of Plate V, Fig. 12, in which they lie in attitudes closely resembling those previously encountered in the fern (Manton and Clarke, 1951*a*) and like the fern suggesting a slow spiral of not more than about two complete turns (four half-turns) in the length of the cilium. Unlike the fern, however, this material shows part at least of the structural mechanism by which the tube is held together.

Our attention was arrested by an appearance as of cross-banding most conspicuously seen in the middle region of the hind flagellum of the cell of Plate V, Fig. 11, which is shown at a higher magnification in Plate VI, Fig. 15. At a first glance this appearance suggests the type of cross-banding revealed by the electron microscope in animal fibres of muscle or tendon, but it is clear from other parts of the cilia on the same cell that this analogy is illusory. The cross-banding is not caused by a discontinuity of structure within the

fibres themselves but is due to the presence of another substance laid across them on the inside of the tube. This other substance is not a homogeneous tube-lining but is in the form of distinct bands separated by slight spaces. There is a possibility that the bands themselves may be multipartite, though this has not yet been explored in detail. The cross-banding runs in straight, or slightly diagonal, lines across the tube fibres where these are still in lateral contact. Where the tube fibres are beginning to fall apart, the material of the cross bands may become pulled out into very delicate threads which lie across the spaces between adjacent fibres like the rungs of a ladder (Plate VII, Figs. 16 and 17). When these have broken the remains of the banding substance may give a curious beaded or battlemented appearance on one side only of a fibre, this side being doubtless its inner surface (cf. Plates V, VI, VII, Figs. 12, 14, and 18). The banding substance can, however, disappear completely, leaving the fibres bare as in other parts of these figures.

All the figures in Plates VI and VII are from the same cell (that of Plate V, Fig. 11), the direct prints contained in Plate VI being from the hind flagellum and the reversed prints of Plate VII being from the front flagellum. Once these appearances have been seen, however, they can be recognized in many other instances and further information can be added. Thus Plate V, Fig. 13 is informative in showing stages in the decomposition of the banding substance. The cilium has become fully dismembered and in places the battlement effect previously described can be made out (see arrows). In most places, however, the material of the 'battlements' is liquifying and becoming spread upon the formvar, although it had not completely washed away before the specimen was dried. This tendency for liquifaction of the banding substance to occur long before the main fibres of the cilium decay is no doubt one reason why the cilium itself is liable to fall apart after death of the cell. The rapidity with which this liquifaction occurs must also be the reason why we have not previously been made aware of this additional material although we have had signs of it in other organisms (for details, see Discussion) but only of a kind which could not be fully interpreted.

Since the banding material is on the inside of the tube where it cannot be seen until dismemberment has begun it is not possible to describe its complete path with certainty. The banding and the 'battlements' are, however, far too regular to be laid down at random, and it is probable that in the intact cilium they are either in rings going right round the inside of the tube or in a flat spiral. The tendency for the bands to appear slightly diagonal, as for example between the arrows on Plate IV, Fig. 10, suggests the latter.

The banding material does not extend quite to the tip of the flagellum, but ends abruptly at a level corresponding to the base of the whiplash point. This is also approximately level with the tips of the shortest fibres. This can be clearly seen in a specimen such as that of Plate IX, Fig. 22. The long fibres, which may include one, though not necessarily both, of the components of the central pair (cf. Plate VIII, Fig. 20) extend some distance farther to form the whiplash point itself.

STRUCTURE OF THE WHIPLASH POINT

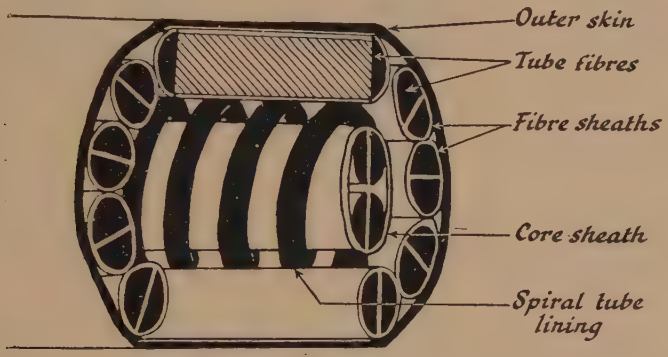
The whiplash point in this material is the slightly transparent apical extremity of the intact cilium in which it narrows to a point unless artefacts intervene. The pointed condition can be seen in both ultraviolet photographs (Plate I, Figs. 1 and 2), in both cilia of Plate III, Fig. 8, and in various parts of intact cilia included incidentally in Plates II and V, Figs. 6 and 13. Both the narrowing and the transparency can now be attributed primarily to the absence of the banding substance last described. The number of strands is also gradually reduced as those of different length come to an end. Each strand is also abruptly reduced in width as it enters the whiplash by a disappearance of what appears to be a delicate sheath round each, comparable in some respects to the common sheath round the central pair of strands (except at their tips) which was described on p. 268. Evidence for this sheath and of the structure of the core of each fibre where it projects beyond the sheath is contained in Plates VIII–IX, Figs. 20–22. In each of the fibres long enough to extend into the region of the whiplash (and this applies to the components of the central pair as well) the naked distal extremity of the strand appears to be double. The doubleness is expressed by the presence of a fine line running along the centre of each fibre as if two parts were lying in contact in the manner shown on a larger scale by the undoubtedly separate central strands of Plate II, Fig. 7. The evidence for the existence of a separate sheath enclosing each fibre lower down on the cilium is also much as in Plate II, Fig. 7, but on a smaller scale. As the eye passes down a fibre in Plates VIII–IX, Figs. 20–22, the signs of doubleness are suddenly obscured when the base of the whiplash is reached and at this point the shadow of the fibre becomes wider, showing that its bulk after drying is greater than in the whiplash itself. The point at which this occurs is the same for all strands no matter how much or how little of them extends into the whiplash, and it seems to correspond to the region at which the cross-banding substance also disappears.

In the intact whiplash the fibrils which extend into it are almost certainly in close lateral contact with each other in spite of the absence of the banding substance, because mutual cohesion in this region is often still maintained (cf. Plates V and X, Figs. 13 and 23), even after the rest of the cilium has fallen apart. One must therefore suppose that in life the core of the whiplash is compact. It is, however, certainly covered by a skin and this skin is also present all over the surface of the cilium. It can best be demonstrated in the somewhat altered apices such as those illustrated in Plate X, Figs. 24–25. In Plate X, Fig. 24 the core of the whiplash is still intact but a blister has formed under the skin farther back, thereby revealing the presence of the skin very clearly. In Plate X, Fig. 25 the skin covering the whiplash has become distended into a vesicle presumably by the liberation into it of the products of decomposition or of other osmotically active liquid after the internal fibrils have separated. This appearance has previously been seen by us in *Marchantia* (Manton, 1951) and has also been described in a number of other bryophytes

by visual microscopists (notably Miduno, 1934). In view of the series of stages from normal to completely decomposed which we have studied here we now regard such vesicular ends as either abnormalities or necrotic artefacts but not as the normal structure of the live cell.

DISCUSSION

The observations on *Sphagnum* described above are sufficiently complete to permit, for the first time, of an attempt to be made to construct a model of an intact cilium. This is shown diagrammatically in Text-fig. 1. The various components (outer skin, fibrils, fibril sheaths, and spiral inner lining to the tube) have all been demonstrated in the electron micrographs quoted, as has the doubleness of the fibrils themselves, at least in their distal extremities.

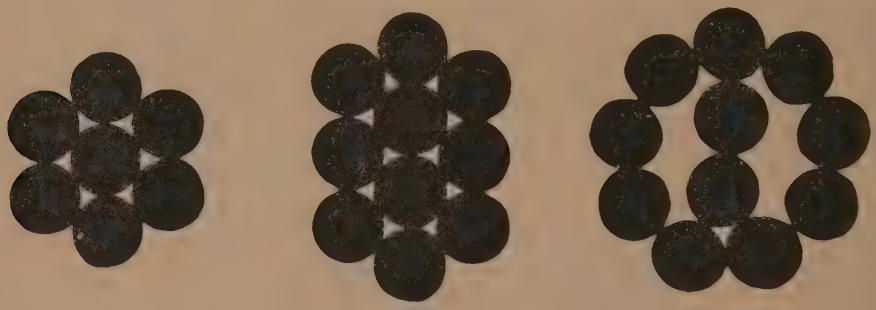


TEXT-FIG. 1. Diagrammatic reconstruction of the cilium of *Sphagnum*. For further explanation, see legend to Text-fig. 2 and text.

One important item is, however, uncertain, namely the shape of the sectional area of the cilium and of the component strands. It is reasonable to assume that the cilium itself is cylindrical and therefore circular in cross-section; this is, however, unlikely to be true for all the components. If, for example, the component strands are also cylindrical, then the central pair at least would be expected to be oval. On the other hand, if this is in any way compressed, the two component strands would be likely also to be compressed in one direction or another. The same is true of the components of the outer tube. It is possible that they are wholly unstressed in life and cylindrical; it is equally possible that they are under either tension or pressure, though there is at present no means of determining this. It cannot therefore yet be known whether their sectional area is circular, wedge-shaped, flattened tangentially, or of some other form. Another detail of uncertainty is the relative position of the split down the centre of each strand. It seems likely to lie radially, but some other position may in fact be the truth, and we have no means of knowing the mutual orientation of the split in the two components of the central double-strand.

In spite of these elements of doubt, however, this diagram is a very great advance on previous knowledge, and it is certainly applicable to other ciliary

types besides that of *Sphagnum*. The reasons for this statement are partly the clarification which the diagram offers for the hitherto baffling regularity of recurrence of exactly eleven strands among all types of plant cilia examined,[1] but also by the recognition that some at least of the new observations here recorded for *Sphagnum* can in fact be repeated in some of our own published micrographs which at the time of publication could not be fully understood.



(*a*) (*b*) (*c*)

TEXT-FIG. 2. Geometrical shapes obtainable by combining various numbers of strands of uniform diameter. Fig. 2*a*, one central strand can accommodate six peripheral strands in contact with it. Fig. 2*b*, two central strands can accommodate eight peripheral strands. Fig. 2*c*, with nine peripheral strands contact with centre is lost and an internal space results. This condition approximates to that of a cilium except that in a cilium the central strands are thinner than the others and the space between core and periphery is correspondingly greater (cf. Text-fig. 1).

Thus Fig. 13 of our paper on *Pylaiella* (Manton and Clarke, 1951*b*) shows exactly the same ladder-like cross fibrils as those illustrated in Plate VII above, but without the other information regarding cross striations discussed on pp. 268–9 we could not explain their nature. It is now clear, however, that Fig. 13 of *Pylaiella* must represent the same effect of lateral tension on cross bands as has been found in *Sphagnum*, and since in *Pylaiella* we are dealing with a *Flimmergeissel*, a morphological form which is as different as possible from the smooth flagella of the Bryophyta, we may confidently expect that similar direct evidence of inner identity of structure will be multiplied.

Assuming this to be true, it is obvious that the regularity of recurrence of eleven strands among cilia of very diverse origins is quite simply explained by the geometry of the structure. That this would be so has long been obvious (cf. for example, Manton, 1951), although it has not hitherto been possible to define the particular geometrical properties involved. It had been expected that a cilium would be a fairly solid structure, in which case a single central strand can only have six others of comparable diameter surrounding it (Text-fig. 2*a*). With two central strands the maximum number of peripheral strands which can be simultaneously in contact with the centre will be eight (Text-fig. 2*b*) and the outline will then be oval. Since the peripheral strands

[1] With the one exception of *Fucus* hind flagellum (Manton and Clarke, 1951*c*), which needs re-examination (for discussion see Manton, 1951).

are invariably nine, it was obvious that they could not all abut directly on the central pair and that there must be some kind of space between the peripheral tube and the core (Text-fig. 2*c*). We now know that this space is occupied by the cross-banding material which forms an inner lining to the tube composed of spiral or circular bands, at least in the two cases of *Sphagnum* and *Pylaiella*. These bands are attached to the inner side of the fibres composing the tube, but they are not attached to the central pair of strands although they probably touch it. If this material is an essential component in all cilia in which eleven strands have been found, then it is clear that nine is the smallest number of peripheral strands which will leave any room for it. On the other hand, if close packing is also essential for effective ciliary motion, it does not need much experimenting with the model to convince oneself of the very great changes in compactness and in the stresses and strains set up in the various parts if even one additional strand is added. It is therefore virtually certain that the absence, from any of the plants we have studied,[1] of any number higher than nine peripheral stands surrounding the central pair must be due to some purely mechanical consideration in the physical functioning of the organ, which controls variability in the upward direction just as rigidly as the geometrical needs of constructing it prevent variation in the downward direction.

We hope, in a final paper now in preparation on the green algae, to amplify some of these statements and perhaps to summarize them more fully. In the meantime, the evidence from *Sphagnum* has not merely added the Bryophyta to the list of groups (Pteridophyta, fungi, brown algae, yellow-green algae, and green algae) which have been studied in the series of papers listed below, but has provided an essential piece of new information regarding what is probably a fundamental feature of construction of the cilia in all these groups.

[1] The list so far published is: Ferns (Manton and Clarke, 1951*a*), *Pylaiella* (Manton and Clarke, 1951*b*), *Fucus* (Manton and Clarke, 1951*c*), *Chlorosaccus*, *Saprolegnia*, *Allomyces Olpidium* (Manton, Clarke, Greenwood, and Flint, 1952), *Ulothrix* (Manton, 1951).

PLATE I

FIG. 1. Spermatozoid of *Sphagnum acutifolium* agg. sp. 1. Ultraviolet photograph (192. 6*b*) of a partly expanded specimen killed with osmic vapour and examined in water. Magnification ×3,000.

FIG. 2. Two other cells of the same, one partly expanded the other in an unnatural attitude, selected to show the head and cilia. Ultraviolet photograph (193. -2*a*). Magnification ×3,000.

FIG. 3. Spermatozoid of the other species (*S. acutifolium* agg. sp. 2) with uncoiling only just beginning; killed with osmic vapour, dried, and shadow-cast with gold palladium. Low-power electron micrograph M79. 13, 60 kV.; magnification ×3,000.

PLATE II

FIG. 4. Spermatozoid of *S. acutifolium* agg. sp. 2. Electron micrographs M71. 2, 4, and 5; magnification ×5,000.

FIG. 5. Tip of the tail of another specimen to show fibrillar appendage. Electron micrograph M78. 27; magnification ×10,000.

FIG. 6. Another cell. Electron micrograph M76. 25 and 27; magnification ×5,000.

FIG. 7. Tip of the central pair of strands from the dismembered flagellum of the cell of Fig. 6, more highly magnified. Electron micrograph M77. 19; magnification ×20,000.

PLATE III

FIG. 8. Partially uncoiled spermatozoid of *S. acutifolium* agg. sp. 2 with both cilia intact. Electron micrograph M72. 20; magnification ×5,000.

FIG. 9. Head of another cell showing attachment of the two cilia, &c. Shreds of the enveloping membrane still visible lying across the intact cilium. Electron micrograph M76. 23; magnification ×10,000.

PLATE IV

FIG. 10. Another cell: the body is still coiled within the enveloping membrane in which a striated structure is visible; both cilia are extruded and showing almost complete fibrillar disintegration. Electron micrograph M78. 2; magnification ×8,000.

PLATE V

FIG. 11. Spermatozoid of *S. acutifolium* agg. sp. 2. Low-power view of the cell of Fig. 12 and of all the figures on Plates VI and VII. Electron micrograph M75. 8; magnification ×3,000.

FIG. 12. Enlarged detail of the head and front flagellum of the cell of Fig. 11 (further details of the front flagellum are in Plate VII). Electron micrographs M77. 14, 15, and 16; magnification ×8,000.

FIG. 13. An intact and a dismembered flagellum from another cell. Electron micrograph M71. 23; magnification ×7,500.

PLATE VI

FIG. 14. *S. acutifolium* agg. sp. 2. Tip of hind flagellum of the cell of Fig. 11. Electron micrograph M75, 11; magnification ×20,000.

FIG. 15. Part of the central region of the hind flagellum of the cell of Fig. 11, showing cross-banding. Electron micrograph M75. 14; magnification ×20,000.

PLATE VII

FIG. 16. Basal part of the front flagellum of the cell of Figs. 11 and 12, showing cross-banding under tension. Electron micrograph M75. 23, reversed print; magnification ×20,000.

FIG. 17. Part of the central region of the front flagellum of Fig. 12 showing cross-banding under tension. Electron micrograph M75. 20, reversed print; magnification ×20,000.

FIG. 18. Part of region near tip of the front flagellum of Fig. 12 showing cross-banding ruptured but residual material still adhering to parts of three fibrils. Electron micrograph M75. 17, reversed print; magnification ×20,000.

PLATE VIII

FIG. 19. *S. acutifolium* agg. sp. 2. Partially dismembered cilium showing some of the tube fibrils, banding substance, and central double-strand still in position. Electron micrograph M79. 25; magnification ×20,000.

FIG. 20. Tip of a dismembered cilium to show detailed structure of the distal extremities of the strands. Electron micrograph M79. 22; magnification ×approx. 15,000.

PLATE IX

FIG. 21. Tips of some of the fibrils of the upper flagellum in Fig. 10 to show details of terminal structure. Electron micrograph M79. 18; magnification ×20,000.

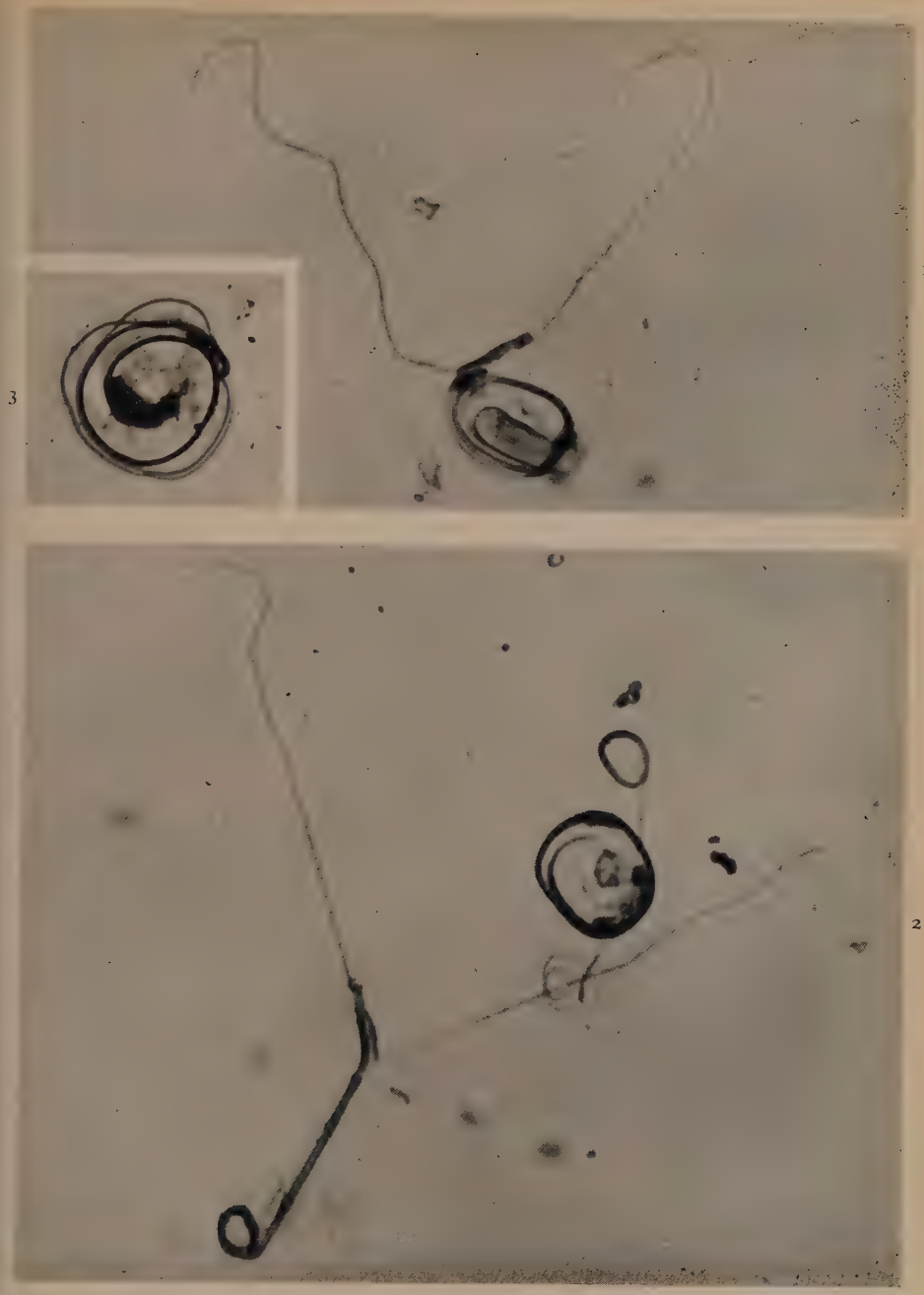
FIG. 22. Part of a similar specimen to show termination of the banding substance. Electron micrograph M79. 28; magnification ×15,000.

PLATE X

FIG. 23. *S. acutifolium* agg. sp. 2. Tip of a dismembered cilium showing the persistent adhesion of the distal extremities of some of the longer strands in the region of the whiplash point. Electron micrograph M78. 13; magnification ×10,000.

FIG. 24. An almost intact cilium from a preparation stripped from glass, showing local raising of a skin covering the outside of the cilium. Electron micrograph M76. 20; magnification ×10,000.

FIG. 25. Tip of a cilium showing local distension of the membrane covering the whiplash point, after loss of cohesion of the internal strands. Electron micrograph M77. 29; magnification ×10,000.



I. MANTON and B. CLARKE—PLATE I

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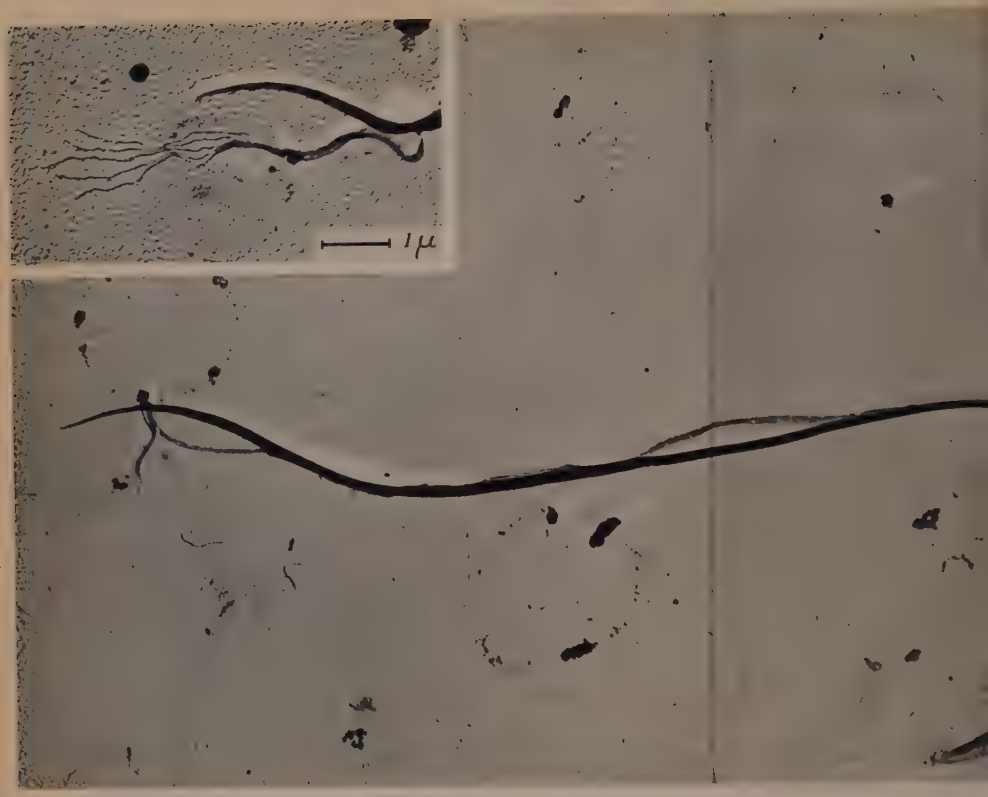

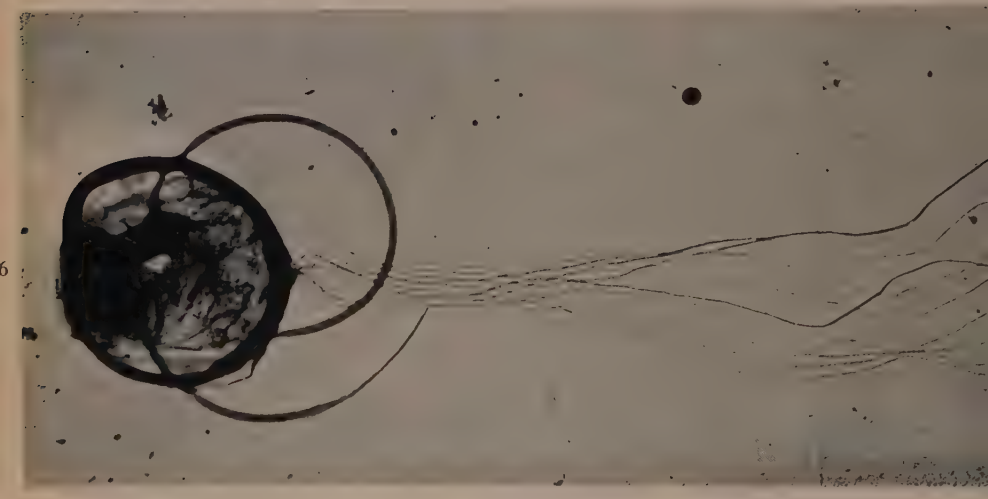

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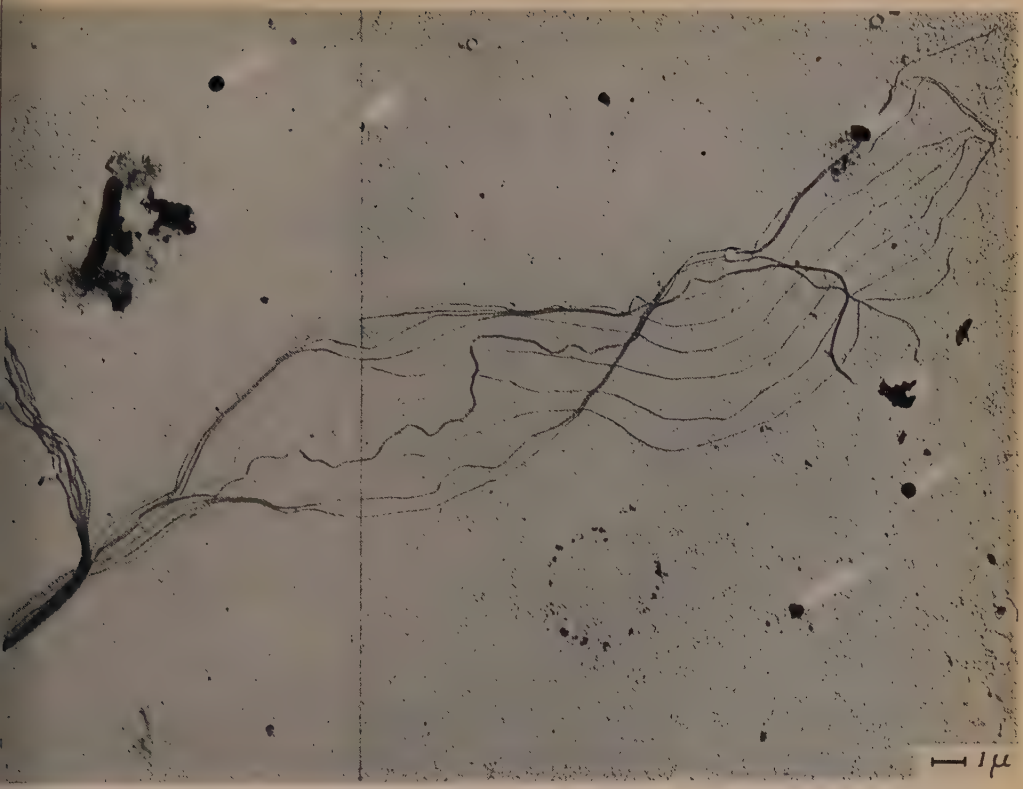

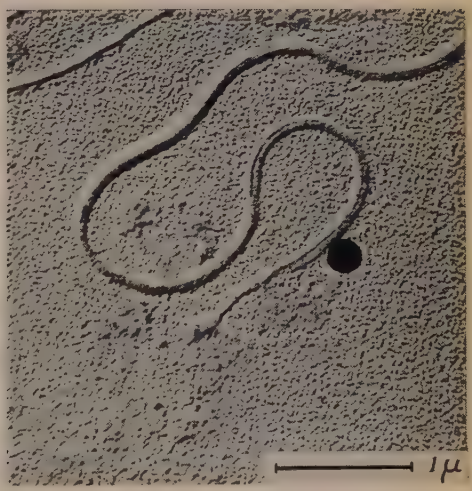

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I. MANTON and B. CLARKE—PLATE III

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I. MANTON and B. CLARKE—PLATE IV

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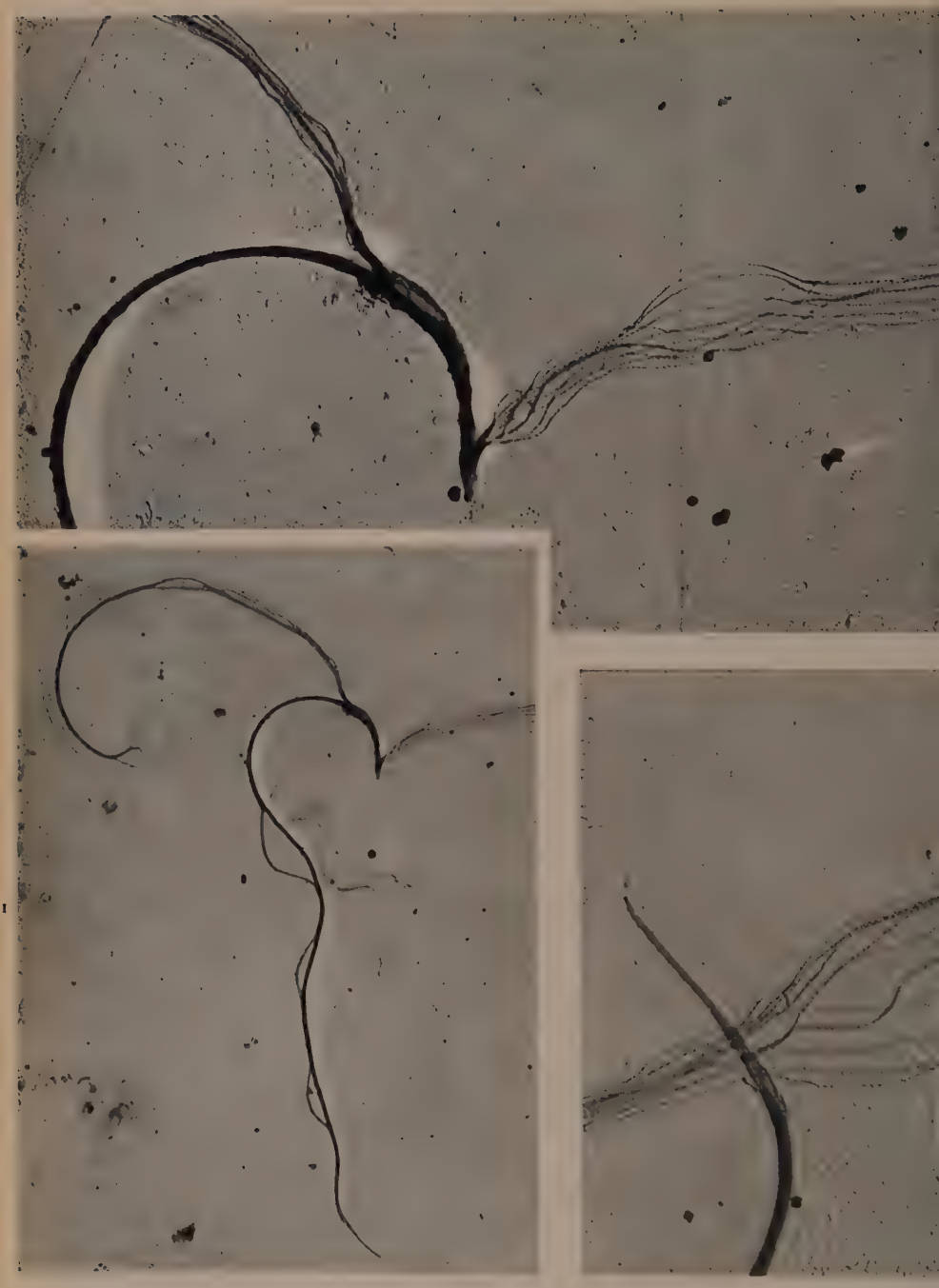

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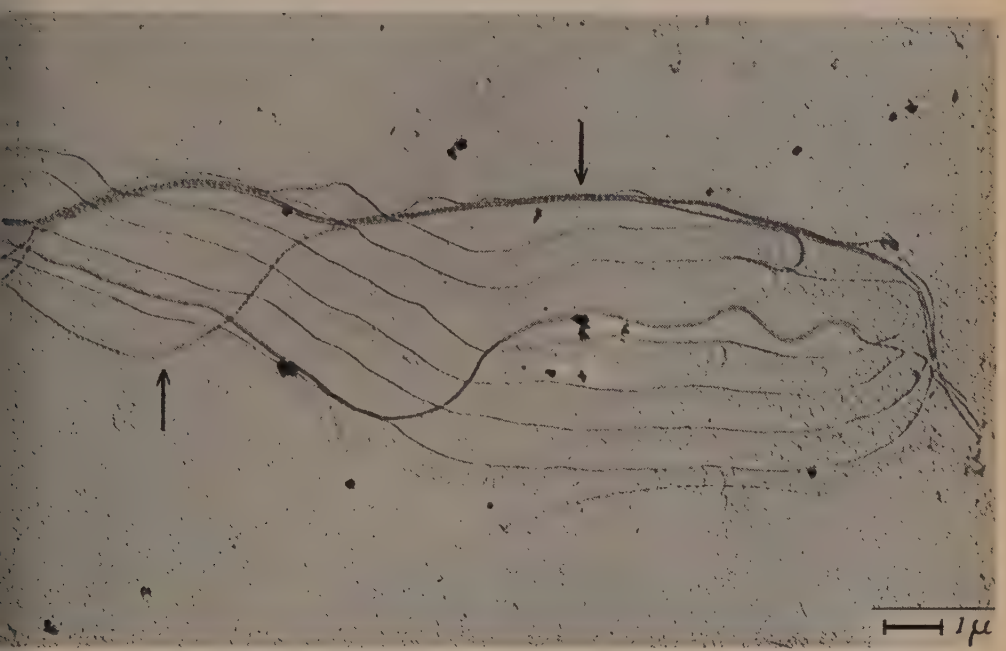

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I. MANTON and B. CLARKE—PLATE VI

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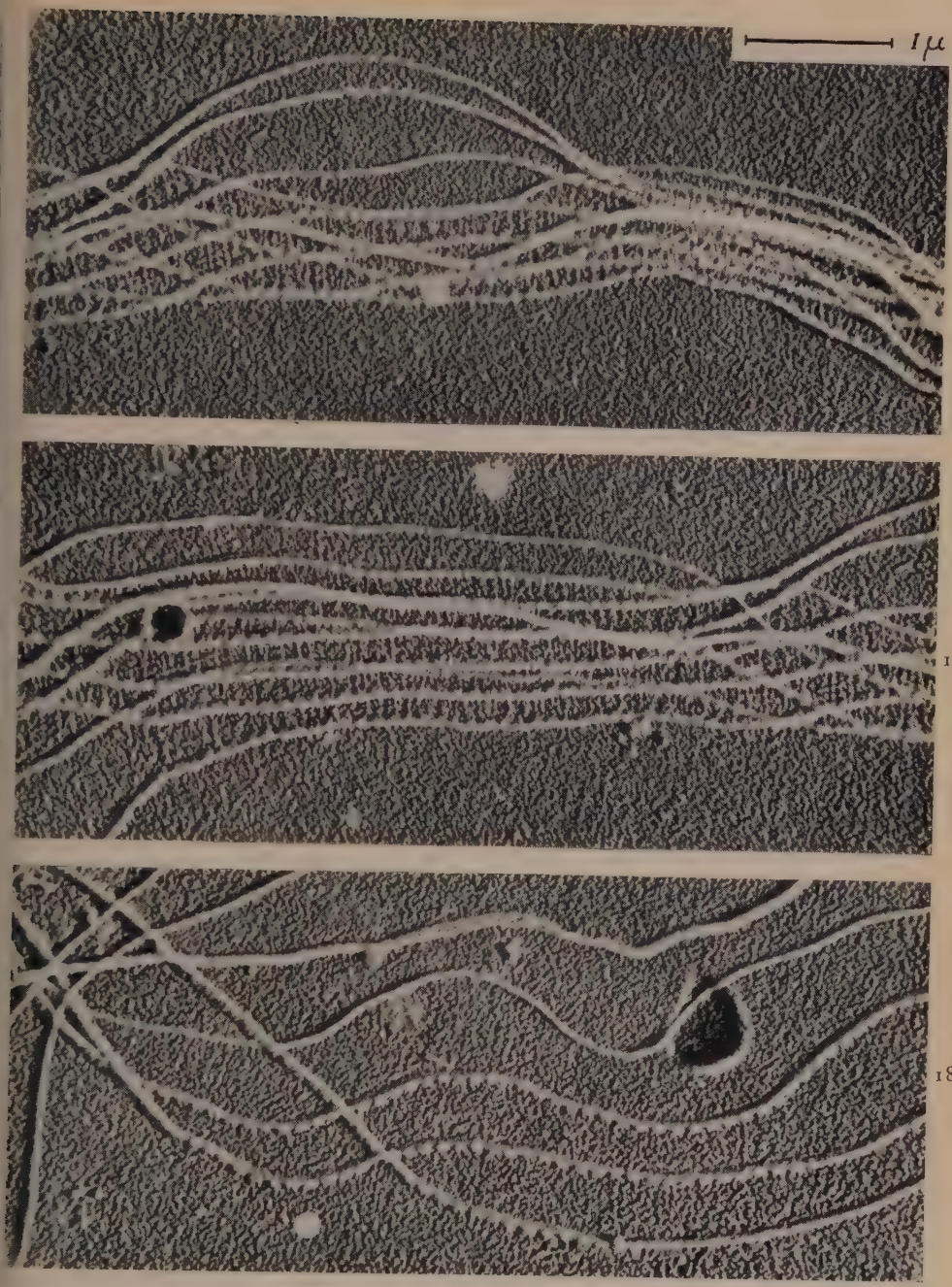

I. MANTON and B. CLARKE—PLATE VII

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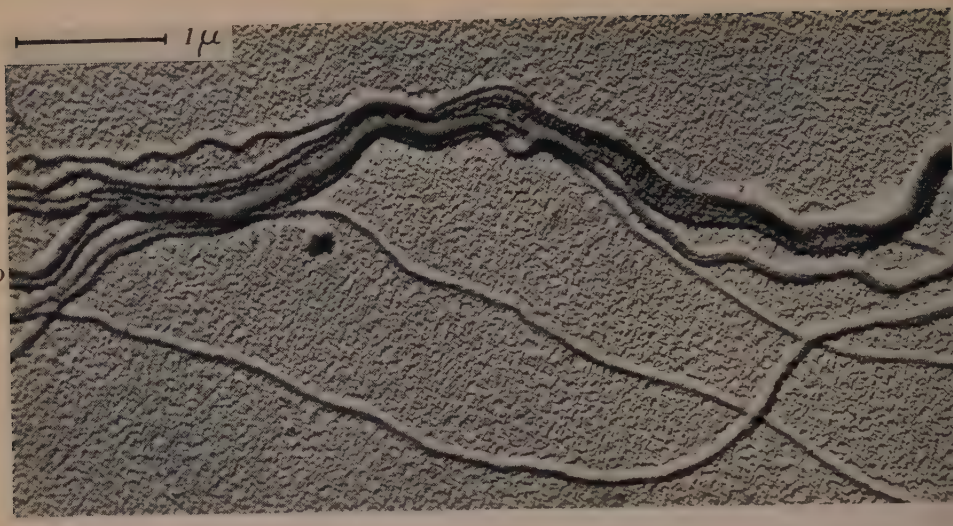

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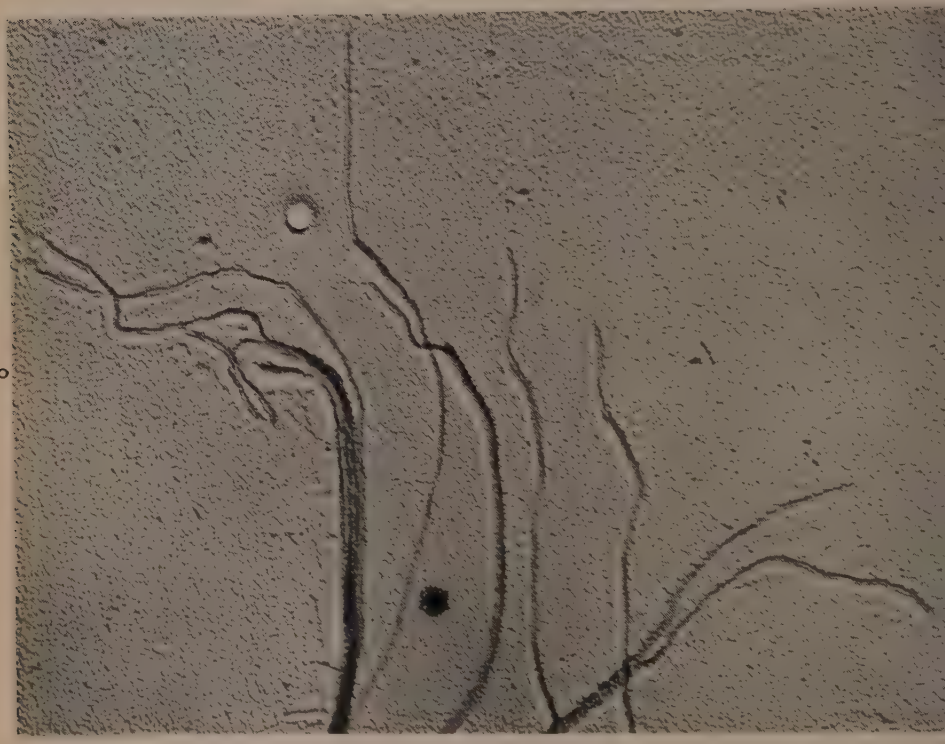

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I. MANTON and B. CLARKE—PLATE VIII

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I. MANTON and B. CLARKE—PLATE IX

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I. MANTON and B. CLARKE—PLATE X

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The Relation of Water Absorption by Whea Seeds to Water Potential

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SUMMARY

The progress of water absorption by wheat grains was studied by supplying water in the vapour phase, at controlled potentials.

At a potential of −250 metres of water, the curve for water uptake against time shows exponential approach to equilibrium moisture content. Living and dead seeds behave similarly until germination effects are apparent. Water uptake in the early stages is probably due to physical rather than physiological processes. When germination occurs, it causes an exponential increase in the rate of water uptake.

At higher potentials, up to zero, the uptake curves for dead seeds depart from the simple exponential relationship; in addition to the exponential component, there is a component the rate of which increases with time to a constant rate. The first component may represent the physical process of imbibition by the starch, and the second the initiation and progress of starch hydrolysis.

A parameter a of the formula derived for the curves is interpreted as representing the diffusivity of water vapour in the seed material, depending upon the physical properties and dimensions of the seed.

INTRODUCTION

When a seed is sown in the soil, the time it takes to germinate depends the rate at which it takes up water, which must be determined partly by pr perties of the seed, and partly by the forces tending to retain water in the so

Slosson (1899) showed that the presence of salts in solution hinders t absorption of water by seeds, and Atkins (1909), Shive (1916), and Rudo (1921, 1925) demonstrated that this effect upon water uptake depends on t concentration of the solutions. Wolfe (1926) showed that all the increase seed weight resulting from immersion may not be due to water uptake, the seeds absorbed some solute which could be washed out with water. Brov (1932) gave data for absorption of both water and sodium chloride by whe grains immersed in solutions, showing that evidently both the nature and t concentration of the salts influence the rates of water absorption. A simi conclusion was reached by Hayward and Spurr (1943, 1944) when consideri entry of water into roots.

The object of the work described in this paper was to measure the effect varying water potential on the rate of water uptake by seeds. It is difficult control the water potential in soil at prescribed values, and for precise expe ments simpler systems are necessary. The most obvious technique, that

immersing the seeds in solutions of varying concentrations, is inadvisable, because of possible complications arising from solute uptake.

In a previous paper (Owen, 1952) a technique and apparatus was described enabling controlled water potentials to be applied to seeds by supplying all the water in the vapour phase. The seeds were held at a controlled temperature in the atmosphere of a confined space above solutions of known proportional vapour pressure lowering, so that the water potential in the environment of the seed was dependent upon the relative humidity.

EXPERIMENTAL

Plant material. The seeds[1] used were of wheat, var. 'Squareheads Master 13/4', harvested in 1948, obtained from the National Institute of Agricultural Botany. To minimize variation between seeds those weighing between 40 and 60 mg. were selected. An organic mercurial dust was used to reduce infection. Before use the seeds were stored over a saturated solution of sodium nitrite at 20° C. for 24 hours, that is, brought to equilibrium with an atmosphere of 66 per cent. relative humidity.

Apparatus. A description of the apparatus and the means for provision of the very precise degree of temperature control necessary are given in a previous paper (loc. cit.), together with the calculation of water potential from the vapour-pressure lowering.

Preliminary Experiment 1. The experiment consisted of a comparison of water uptake by intact seeds and seeds from which the embryos had been removed. Four potentials, −160, −205, −252, and −298 metres of water, corresponding to concentrations of sodium chloride of 0·35 M., 0·45 M., 0·55 M., and 0·65 M. respectively, were used. Observations were made at 4 sampling times at intervals of 4 days.

320 seeds were selected and from 160 of them the embryos were cut away and discarded, care being taken to remove as small a portion of endosperm as possible. The cut surface of the seeds was lightly smeared with vaseline.

The weighed seeds were placed in batches of 5 in the controlled humidity tubes at 20° C. After the appropriate period in the thermostat bath at 20° C., two tubes of seeds at each concentration, for both intact and 'excised' seeds, were removed. The seeds were transferred to previously weighed stoppered bottles, weighed immediately, dried at 105° C. for 3 days, and reweighed. The water content as per cent. of dry weight for each batch is shown in Fig. 1.

The mean dry weight of an excised seed was 39·5 mg. and that of an intact seed was 46·8 mg.; therefore 100 g. of intact seed consisted of $100 \times \frac{39{\cdot}5}{46{\cdot}8} = 84{\cdot}3$ g. endosperm and 15·7 g. of embryo, ignoring the small amount of endosperm removed with the embryo. An estimate of the water content of the endosperm as per cent. of dry matter of the whole seed was

[1] The term 'seed' is used throughout this paper to denote the wheat caryopsis.

therefore obtained by multiplying the water as per cent. of dry matter of t excised seeds by 0·84. The difference between this and the water content p cent. of the intact seed gave the water content of the embryo.

Fig. 1, B shows that the rate of increase of water content for the endosper

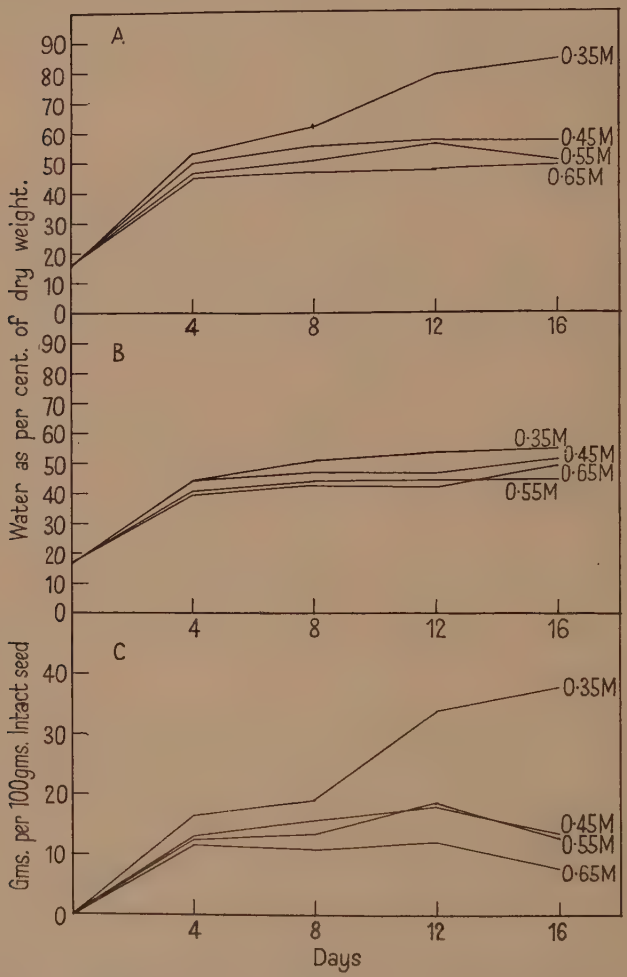

FIG. 1. Preliminary Experiment 1. Increase in water % of dry weight. A, Intact seeds; B, excised seeds; C, estimate of water uptake by embryo.

decreased with time until an equilibrium value was reached. In Fig. 1, A t intact seeds at the highest potential continued to increase in weight throughc the duration of the experiment, as embryo activity increased. The contrib tion made by change in weight of the embryo to that of the whole seed shown in Fig. 1, C. Comparison of A, B, and C in Fig. 1 indicates that the eff

of the water uptake by the embryo began to be apparent at the first sampling time, and decreased with increasing solution concentration, i.e. decreasing water potential. The continued rise in water content of the whole seed held over 0·35 M. salt solution was evidently due to water uptake by the embryo, associated with germination.

Preliminary Experiment 2. Although the dimensions of the seed used were standardized as much as possible by selection, a certain variation was inevitable. Variation in the internal diameter of the paper tubes and the difficulty in placing the seeds exactly on the central axes of the tubes led to varying air gaps around each seed. The resistance to diffusion of this gap is one of a number of resistances in series and it was desirable to know whether variation of this resistance had any appreciable effect on the rate of water uptake. This was tested in Expt. 2 by comparing paper tubes of different internal diameters, viz. 6 mm. (normal tubes, *N*) and 10 mm. (wide tubes, *W*).

As the average seed diameter was 4 mm., the mean gap widths for *N* and *W* were respectively 1 mm. and 3 mm. Samples were taken by the same procedure as Expt. 1 daily for 5 days to give more detailed information on the early stages of uptake. The results (Fig. 2) and the analysis of variance (Table I) show that variation of the air gap had no significant effect.

TABLE I

Analysis of variance, Expt. 2

Variance due to	D.F.	Mean square	Variance ratio	Probability
Time (*T*)	4	252·2	100·9	< 0·001
Gap width (*G*)	1	0·5	—	—
Conc. NaCl (*C*)	2	141·8	56·7	< 0·001
C×*G*	2	0·05	—	—
T×*G*	4	3·8	1·5	—
T×*C*	8	2·8	1·1	—
T×*C*×*G*	8	0·5	—	—
Error	30	2·5	—	—

The experimental variation in gap size of 3 times the normal width was much larger than could occur by chance in the other experiments, so the effect of chance variations can be ignored. Calculation of the theoretical rate of transfer of water, assuming the resistance of the air gap to be the only resistance involved, indicates that, with a solution of 0·55 M. NaCl, for example, the seed should reach a moisture content of 36 per cent. in 1 hour. Later experiments showed that the time taken to attain this value was 20 hours, so that the resistance to diffusion of the air gap is negligible. The main resistances involved are probably in the seed coat, and within the seed.

Main series of experiments. In subsequent experiments the progress of water uptake was established more accurately by making observations at shorter time intervals. The procedure was similar to that used for intact seeds in Preliminary Experiment 1. The intervals between samplings were lengthened

as the rate of increase of moisture content decreased. Six concentrations o NaCl were used, from pure water, representing zero potential, to 0·55 M. representing a potential of —251 metres of water.

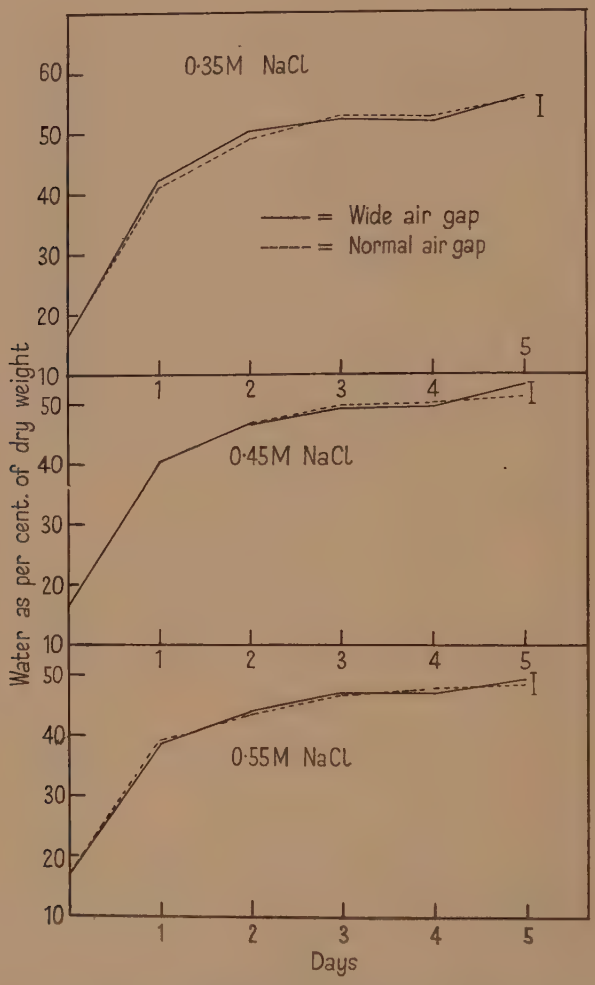

FIG. 2. Effect of air-gap widths. Vertical lines represent least significant difference.

At the higher potentials the effects of the growth of the embryo soon becam apparent. To eliminate this complication, the full series was repeated o seeds the germination of which had been inhibited by exposure to propyler oxide vapour for 8 hours. It was not certain that the inhibition was permanen but for convenience these seeds will be referred to as dead.

The pooled results of all the experiments are given in Table II.

TABLE II

Mean water content as per cent. of dry matter for living and dead seeds. (Data for dead seeds in brackets)

Time (hours)	Concentration NaCl solution (M.)											
	0		0·15		0·25		0·35		0·45		0·55	
2	20·6	(23·0)	20·2	(22·6)	20·6	(22·2)	20·8	(22·2)	20·0	(22·2)	20·1	(23·6)
4	24·7	(26·2)	24·2	(27·2)	23·8	(25·2)	23·7	(26·2)	23·4	(26·2)	23·6	(25·6)
6	27·2	(31·2)	27·3	(30·0)	26·6	(29·4)	26·2	(29·3)	26·8	(28·3)	26·9	(27·7)
8	31·5	(32·7)	30·4	(33·4)	29·3	(33·1)	28·8	(33·5)	28·9	(31·8)	27·8	(30·8)
10	33·7	(35·6)	32·4	(33·5)	32·6	(33·0)	31·1	(34·4)	30·2	(34·2)	30·4	(33·5)
12	37·4	(38·7)	37·0	(36·0)	35·0	(35·3)	34·1	(35·7)	33·5	(35·5)	32·6	(34·3)
14	39·0	(39·7)	37·5	(38·7)	36·7	(37·5)	35·7	(37·6)	34·8	(36·7)	34·4	(36·0)
18	42·8	(42·5)	40·4	(42·1)	39·2	(39·1)	38·2	(40·7)	36·5	(39·8)	35·8	(38·3)
24	50·2	(50·0)	44·4	(45·5)	42·8	(42·5)	41·8	(43·8)	40·3	(41·6)	39·1	(40·7)
36	53·4	(56·4)	50·8	(52·1)	46·7	(49·6)	45·8	(47·6)	45·2	(44·3)	43·0	(46·2)
48	59·6	(59·6)	54·1	(52·9)	51·6	(51·8)	50·5	(49·6)	50·5	(46·8)	44·4	(45·1)
72	66·8	(70·0)	58·2	(62·3)	52·6	(54·7)	51·2	(52·5)	48·7	(49·0)	47·0	(45·9)
96	82·4	(75·5)	62·3	(62·9)	55·7	(58·0)	53·7	(54·2)	49·0	(49·8)	46·4	(48·5)
120	93·7	(78·4)	67·6	(65·7)	58·3	(57·6)	54·3	(56·9)	51·7	(50·9)	48·9	(51·7)
144	121·8	(92·0)	76·0	(66·2)	65·7	(62·4)	55·1	(55·2)	49·1	(57·1)	49·4	(46·9)
168	159·9	(89·4)	97·0	(73·8)	72·1	(59·2)	56·7	(60·4)	51·4	(53·4)	47·5	(50·7)

ANALYSIS OF THE RESULTS AND DISCUSSION

When the water as per cent. dry matter was plotted against time for each concentration it was apparent that the points fell on, or near, smooth curves. The curves for the highest concentration (0·55 M.) exhibited the least difference between living and dead seeds, because at this concentration the living seeds did not germinate within the experimental period. These curves (Fig. 3) were therefore considered first.

The water percentage of both living and dead seeds increased rapidly at first, and then the rate of increase fell as an equilibrium moisture content was approached. As will be shown later, the curves for most of the dead seeds were similar, while the live seed curves, after beginning in the same way, showed a subsequent further increase in water content when germination began.

Form of the water uptake curves. Data in Table II suggest that the rate of increase in water content decreases with decrease in the deficit from the equilibrium moisture content. This suggests an exponential approach to an equilibrium, and the simplest relationship producing such a curve is

$$y = y'(1-e^{-at}),$$

where y is the water content at time t and y' the equilibrium moisture content.

If the initial moisture content of the seed is y^0, this becomes:

$$y'-y = (y'-y^0)e^{-at}. \qquad (1)$$

Hence,
$$\log_e \frac{y'-y}{y'-y^0} = -at$$

or
$$\log_e(y'-y)-\log_e(y'-y^0) = -at.$$

For each concentration, $y'-y^0$ is a constant, so that if this relationship holds, a plot of $\log_e(y'-y)$ against time (t) should give a straight line, the slope of which represents a. Fig. 3 shows such a plot for living and dead seeds. Regression lines fitted to the points gave values for a of 0·055 and 0·062.

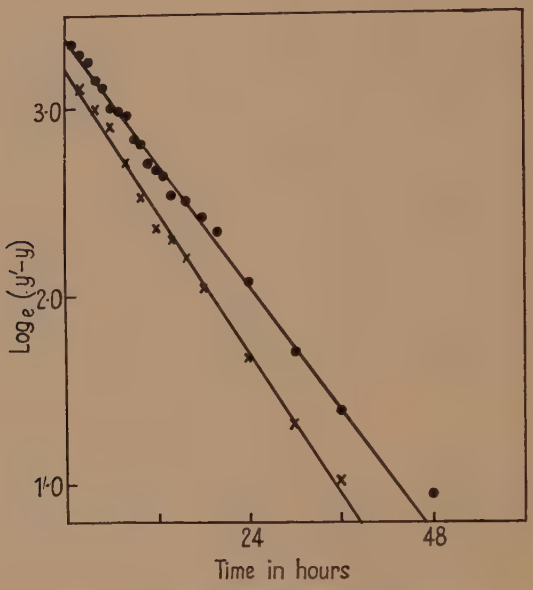

FIG. 3. Water uptake over 0·55 M. NaCl.
Live seeds —●—; dead seeds —×—.

The data for low potentials fit curves constructed from this relationship in a satisfactory manner, but once germination effects have become apparent, the curves for live seeds show rapid increase beyond the asymptotic value. Assuming that the growth of the embryo is exponential, the later portion of such a curve could be represented by:

$$y-y' = (y'-y^0)e^{bt} \qquad (2$$

from

$$y = y'(1+e^{bt}).$$

The complete curve could then be represented by equation (1) up to y and the sum of (1) and (2) beyond. Fig. 4 shows such a complete curve fitted to the data for live seeds at zero potential, and a curve based on equation (1) alone fitted to the data for dead seeds at the same potential. The main interest for the present paper lies in the part of the curve up to the point at which germination effects appear.

The procedure for fitting curves to the data was as follows: (1) An asymptote (y') was chosen by inspection and $y'-y$ was plotted against time on log × linear graph paper. (2) The value of y' was adjusted by steps of 0·5 per cent. water to give a straight line over the greater part of the data. This, o

course, applied only to the earlier part of the curves for living seeds, before germination had begun. Usually only one or two trials were necessary, y' being adjusted through a very small range. (3) The best value of y' having been decided, values of $\log_e(y'-y)$ were plotted against time and the best straight line fitted by the method of least squares. The regression coefficient

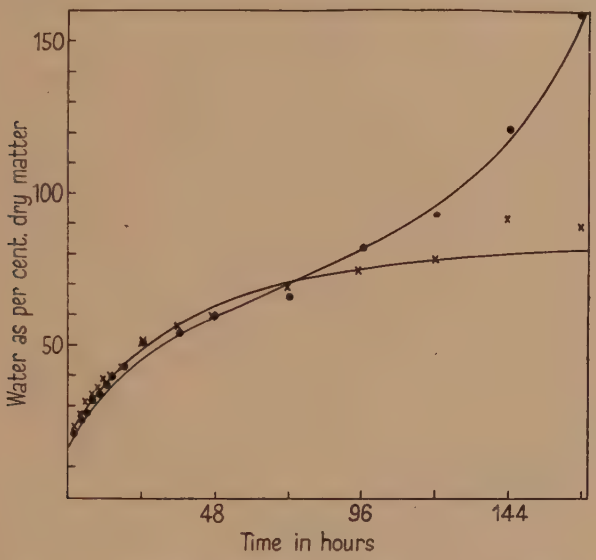

FIG. 4. Calculated curves fitted to data for zero potential.

or slope of the line represented the value of a. (4) From the regression equation $Y = (\bar{Y}-b\bar{t})+bt$, values of Y, representing $\log_e(y'-y)$, were calculated for different values of t, and used to construct the curves relating y to t.

Figs. 5 and 6 show such curves fitted to all the data, and demonstrate the general effect of time and concentration on water content.

For each concentration it would be reasonable to expect the first portion of the curves for living and dead seeds to be substantially the same if the factors controlling uptake at this stage are mainly physical. Comparison of the curves in pairs reveals that there is a systematic difference between them; the uptake curve for dead seeds is similar in form to that for living seeds, displaced horizontally during the first 24 hours. Subsequently the curves converge and remain similar until germination begins. If the time axis of the dead-seed curve is moved laterally by adding 1 hour to each observed time, the two curves coincide satisfactorily. The first inference is that the dead seeds had started from larger initial moisture contents, but this is unlikely, as the initial moisture contents were standardized in all experiments by equilibrating the seeds with an atmosphere of 66 per cent. R.H. An alternative hypothesis is that the propylene oxide treatment altered the initial uptake rate by causing a change in the resistance to water movement offered by the outer layers of the seed. As the moisture content of the outer layers increased, the

effect of their resistance would decrease with time, because the resistances of other regions within the seed would become more important in limiting uptake rate. This would lead to the curves for 'treated' and 'untreated' seed converging.

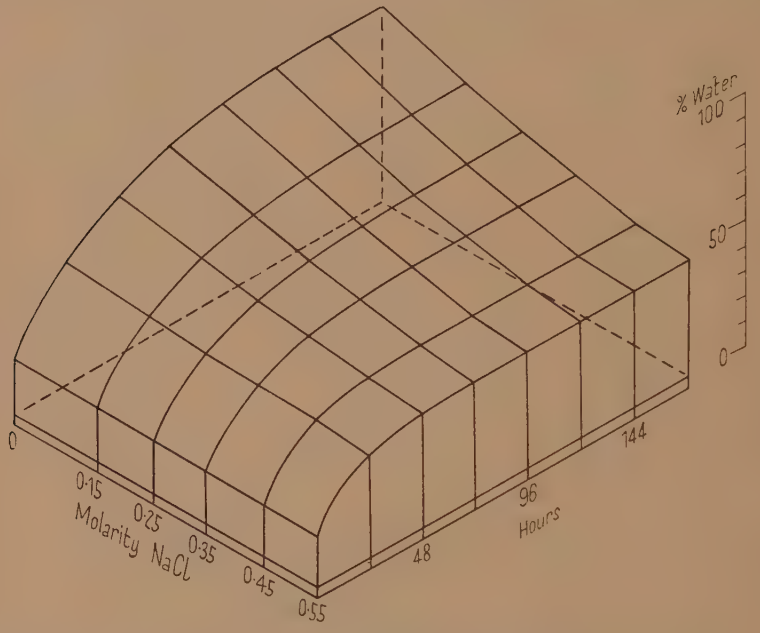

FIG. 5. Influence of time and concentration on water content. (Dead seeds.)

Significance of the parameter a. Water is supplied to the seed in the vapour phase, across an air gap. Preliminary Experiment 2, and the calculated rate of water transfer referred to in the description of that experiment, indicate that the water source may be regarded as effectively maintained at constant strength at the surface of the seed for each water potential studied. In fitting the curves the slope of the lines when $\log_e(y'-y)$ is plotted against time provides values of a in the equation. It is probable that the constant a represents some factor or coefficient concerned with the movement of water or water vapour inward from the surface of the seed.

In the expression e^{-at}, the exponent of e must be a pure number, having no dimensions, therefore a must have the dimensions of a reciprocal of time. Mathematical analyses of diffusion problems of this kind invariably lead to a function of a similar type to a having the form $A = Bk/L^2$, where B is a dimensionless constant, L is a linear dimension of the system and k is a diffusivity coefficient having the dimensions (length)^2/time or $L^2.t^{-1}$.

It therefore seems reasonable to assume that the same is true here, and that a may be regarded as diffusivity divided by the square of some linear dimension of the seed. The seed size was almost constant throughout the

experiments, so that a may be considered to be directly proportional to the diffusivity of water in the seed.

The values of a determined in fitting the curves (Table III) vary with solution concentration or water potential. As potential decreased from zero

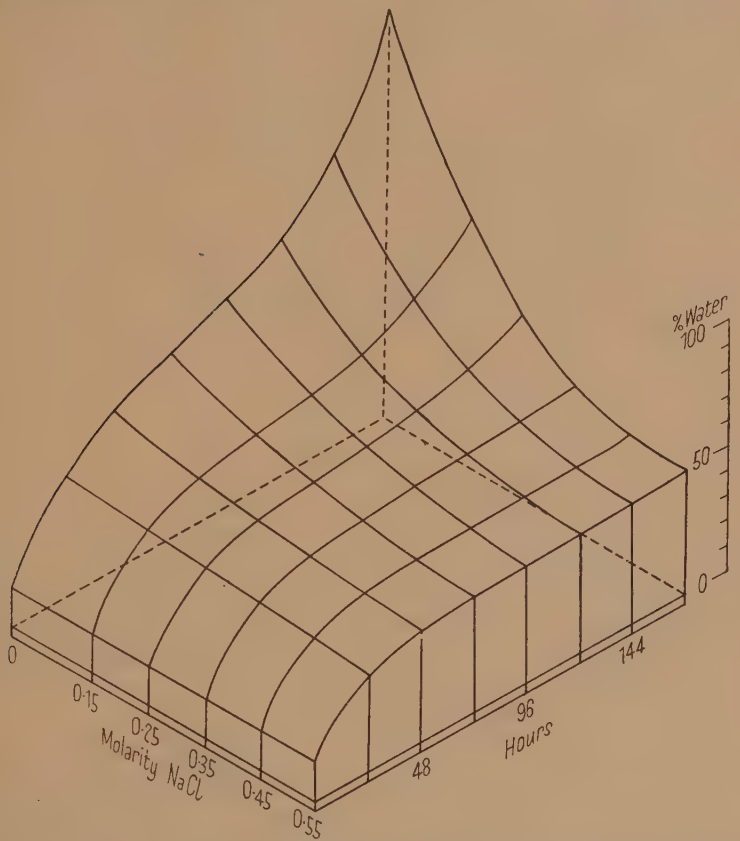

FIG. 6. Influence of time and concentration on water content. (Living seeds.)

to −252 metres of water (0·55 M. NaCl) a apparently increased. The computed values depend, to some extent, upon the choice of asymptote, so that the change in a with potential may be attributable partly to errors in estimation of y', especially at the higher potentials.

TABLE III

Conc. NaCl	Water potential	a (hours^{-1})		y' (%)	
		Live	Dead	Live	Dead
0	0	0·038	0·022	68	82
0·15 M.	−69 metres	0·045	0·031	59	66·5
0·25 M.	−113 ,,	0·047	0·037	54	59
0·35 M.	−160 ,,	0·050	0·046	52	54
0·45 M.	−205 ,,	0·054	0·057	50	49
0·55 M.	−252 ,,	0·055	0·062	47	46

It is reasonable to expect that, if the exponential relationship holds for th full range, the asymptotes for living and dead seeds should be approximatel the same, but Table III shows that, for the values of y' used, this is only tru for concentrations greater than 0·35 M., and the difference between asymp totes for living and dead seeds increases as zero potential is approached. If

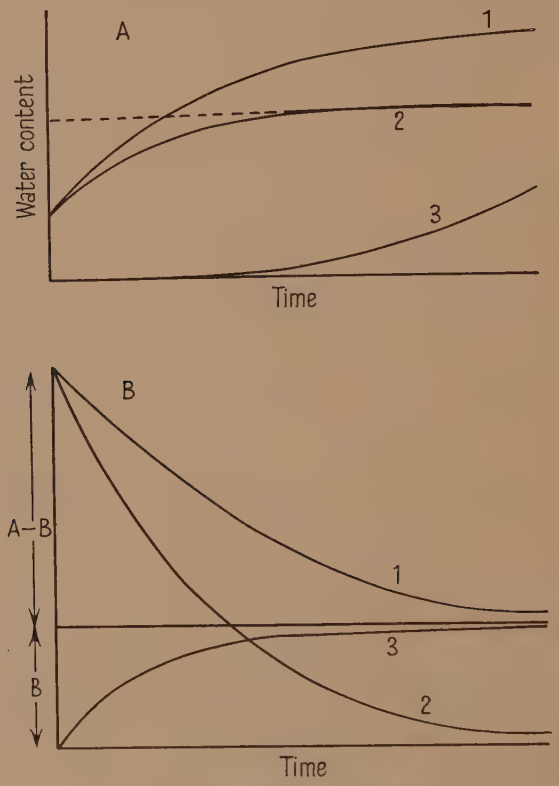

FIG. 7. For explanation see text.

represents diffusivity and is approximately constant along the length of eac curve, its variation with potential as shown above suggests that the simp exponential relationship needs modification at the higher potentials. The shap of the curve for dead seeds over pure water (Fig. 4) suggests that, instead attaining an equilibrium moisture content, the seeds continue to increase i weight, and that the rate of the increase eventually becomes fairly constan This suggests that the uptake curve for high potentials may be made up at least two curves, one representing the physical uptake as suggested f the lower potentials, and one attributable to some other process which, f the period investigated, is initiated only at high humidities.

This hypothesis is illustrated in Fig. 7, A, where curve 1 represents th change with time in water content of dead seeds held at zero potential. Th curve is interpreted as the sum of two components: curve 2, which has th

form given by equation 1 that was an adequate expression for the uptake curves at lower potentials, and curve 3, showing a rate of uptake increasing exponentially with time to a constant rate.

Fig. 7, B shows the rate of change of water content (y) for curve 1 and its components plotted against time.

For curve 2, $dy/dt = Ae^{-at}$, where A is the initial rate of water uptake.

For curve 3, $dy/dt = B(1-e^{-at})$, where B is the constant rate of uptake attained when t is increased indefinitely. Then

$$\frac{dy}{dt}(\text{curve 1}) = \frac{dy}{dt}(\text{curve 2}) + \frac{dy}{dt}(\text{curve 3})$$

$$= Ae^{-at} + B(1-e^{-at})$$

$$= (A-B)e^{-at} + B.$$

Hence, for curve 1,

$$y - y^0 = \int_0^t [(A-B)e^{-at} + B]\, dt$$

$$= \frac{A-B}{a}(1-e^{-at}) + Bt. \qquad (3)$$

A curve of this form was fitted to the data by the following method, illustrated in Fig. 8: (1) A, representing initial uptake rate, is the slope of the tangent to the very early portion of the observed curve. (2) For very large values of t, $e^{-at} = 0$ and equation 3 becomes

$$y - y^0 = \frac{A-B}{a} + Bt.$$

This equation represents the line which the final curve (curve 1) approaches asymptotically, and has slope B and intercepts the ordinate at $(A-B)/a$. (3) Let the point at which the straight lines of slope A and B intersect be P. At point P let the value of y be y_p. On line A, $y_p - y^0 = At_p$. On line B,

$$y_p - y^0 = \frac{A-B}{a} + Bt_p.$$

Whence

$$(A-B)t_p = \frac{A-B}{a},$$

$$t_p = \frac{1}{a} \quad \text{and} \quad y_p - y^0 = \frac{A}{a}.$$

Thus lines A and B intersect at a time equal to $1/a$ hours. This provides a practical test for the fit of the experimental data to the postulated final curve.

If the difference between the asymptote (the B line) and the experimenta curve, at time t, is designated X,

$$X \text{ at time } t = \left[\frac{A-B}{a}+Bt+y^0\right]-\left[\frac{A-B}{a}(1-e^{-at})+Bt+y^0\right]$$

$$= \frac{A-B}{a}e^{-at};$$

$$X \text{ at time } \left(t+\frac{1}{a}\right) = \frac{A-B}{a}e^{-a(t+1/a)} = \frac{A-B}{a}e^{-at-1};$$

$$\frac{X \text{ at time } t}{X \text{ at time } (t+1/a)} = \frac{\frac{A-B}{a}e^{-at}}{\frac{A-B}{a}e^{-at-1}} = e.$$

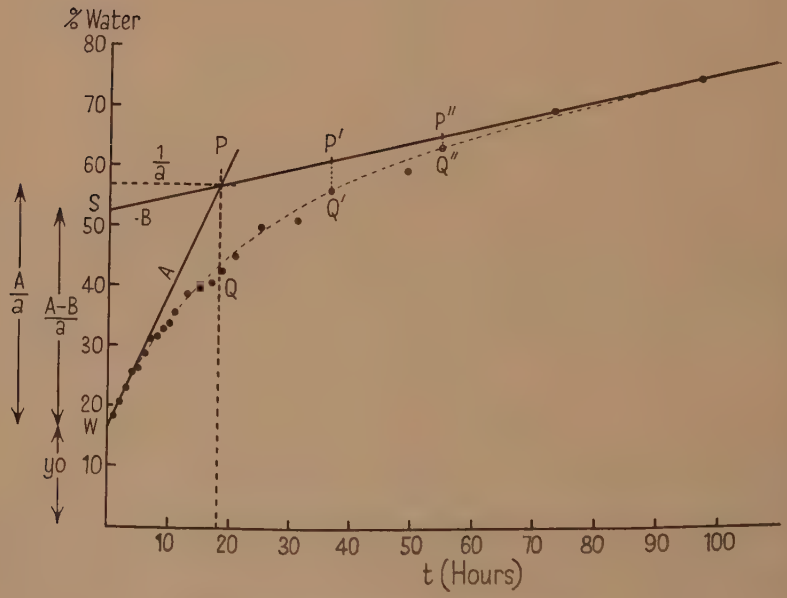

FIG. 8. Composite curve at zero potential.

Thus if point P is at a distance $1/a$ hours from the ordinate, $PQ = (1/e)SW$ where Q is the point where the perpendicular at time $1/a$ cuts the experimenta curve in Fig. 8. The points plotted in Fig. 8 are the data for dead seeds a zero potential. Because the data for both dead and living seeds over 0·55 M NaCl fit a simple exponential curve very well, and the asymptote is easil determinable, the computed value of a for this concentration in Table II is likely to be the nearest to the true value. As there is an apparent effect o propylene oxide treatment on the time axis, of the two values of a at 0·55 M NaCl, that for living seeds is likely to be nearer to the true value. Therefor we take $a = 0{\cdot}055$ and $1/a = 18{\cdot}18$ hours. In Fig. 8, as has been show

above, PQ, representing the difference between the asymptote and a smooth curve drawn through the observed data should be $(1/e)SW$, or the height of the intercept of line B above y^0, because the two are separated by a time interval equal to $1/a$. Also, if at intervals equal to $1/a$ along the time axis the points Q' and Q'' are plotted so that

$$P'Q' = \frac{1}{e}PQ \quad \text{and} \quad P''Q'' = \frac{1}{e}P'Q',$$

these points should fall on the smooth curve through the experimental points.

Fig. 8 shows that points plotted in this way fall on such a curve. The data for dead seeds at 0·15 M. NaCl can also be fitted by a curve constructed by this method, but at lower potentials the simple exponential equation provides satisfactory curves.

To summarize: At low water potentials (—250 metres of water), for periods of up to a week, an exponential relationship of the form

$$y = y'(1-e^{-at})$$

is satisfactory to explain the observed data. (The initial moisture content is ignored here for the sake of clarity.) This suggests that, at low potentials, the entire uptake is due to physical processes.

At higher potentials the divergence of the observed data from the simple exponential curve increases, and it is suggested that the curve for water uptake (excluding germination effects) has an additional component showing exponential increase in uptake rate, the rate of both processes being determined by the same diffusivity constant. The first component probably represents imbibition as suggested above, and the second represents some other process whose initiation is delayed at lower potentials.

This component may be the result of several processes, but one possible explanation is that it is due to sugar production by starch hydrolysis. As potential decreases, the influence of this tentatively designated 'hydrolysis component' becomes less until, at potentials of —250 metres, its contribution to water uptake is negligible in the time studied. In a previous paper on germination (Owen, 1952) it was shown that, at such potentials, wheat seeds germinate in up to 10 days, so that decreased potential apparently only postpones the beginning of hydrolysis, and this may be a possible explanation of the mechanism of the postponement of germination by low water potentials. If correct, this explanation implies that propylene oxide vapour treatment did not prevent enzyme activity in the seed. This is in accordance with observations of Baerwald (1945). As the seeds were not sterile, some of the hydrolysis may have been due to bacterial infection.

The use of the compound curve satisfies the requirement that if a represents the diffusivity of the material of the seed to water vapour, it should not vary with potential. The value of a determined for conditions which are likely to give the most precise estimate, i.e. at low potentials, can be used to account for the change in rate of water uptake in conditions where a is not so precisely

determinable, i.e. at high potentials. This strengthens the view that *a* is pro portional to the diffusivity of the material, and represents a constant property of the seed.

The suggestion that the 'hydrolysis component' is due to starch hydrolysi led to a test of sugar contents of seeds treated with propylene oxide vapou for 8 hours and then exposed to zero water potential in the dark for varyin times up to 6 days. Reducing sugar estimates carried out on seed extracts by colorimetric method (Nelson, 1944) gave the following results:

Days	0	4	5	6
Sugar (mg. per g.)	1·5	1·8	1·6	5·5

Apparently little change in the sugar contents of the seeds had occurre until after the fifth day, when an appreciable increase occurred.

ACKNOWLEDGEMENTS

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The Absorption of Ions by Excised Root Systems

III. OBSERVATIONS ON ROOTS OF PEA PLANTS GROWN IN SOLUTIONS DEFICIENT IN PHOSPHORUS, NITROGEN, OR POTASSIUM

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SUMMARY

1. Pea plants were grown in complete culture solution and in deficiencies of phosphorus, nitrogen, or potassium for a period of about 5 weeks. Excised roots of these plants were treated with a complete, aerated culture solution for varying periods of time and the changes in respiration rate, phosphorus, nitrogen, potassium, sugar, and starch contents measured.

2. There were changes in fresh weight and dry weight of the excised roots during treatment. The dry weight decreased with time but the water content changes were variable. Uptake of water was correlated with uptake of potassium and sucrose content in some instances.

3. There was no evidence of a 'salt respiration' in those cases where active accumulation occurred.

4. The rates of gain or loss of phosphorus, nitrogen, or potassium at 0 hours, 8 hours, and 16 hours were calculated and it was found that the rate depended both on content of element in the root and the sugar content. There was very little evidence that one element affected the rate of uptake of another. Simultaneous loss of one element and gain of another occurred in some instances.

5. The observations appear to be best explained on the assumption that the absorbed ions are fixed in the cells in the form of loosely bound compounds and that these compounds are formed from sugars.

EXPERIMENTAL METHODS AND MATERIALS

In the previous paper of this series (Humphries, 1951) results were presented showing that excised roots of barley plants which had been grown in varying conditions of nutrient supply were capable of absorbing ions from a complete, aerated nutrient solution and the factors determining rate of uptake were discussed. It was also shown that simultaneous loss of one ion and gain of another could occur. The present paper deals with experiments performed on excised roots of pea plants grown under similar conditions of nutrient deficiency.

The procedure for growing the pea plants was similar to that already described for barley. Peas (var. 'Laxtonian') were soaked over-night in running tap-water and put in glass germinators. Two days later when the radicles were 1–2 cm. long the plants were placed on strips of waxed net supported on wooden grids over 2½-gal. tanks (Humphries, 1950, 1951). Thirty-six peas were set out in each of 18 tanks, each strip holding 3 peas. Three separate experiments were performed, viz.: (*a*) *Phosphorus experiment* with three levels of phosphorus supply comprising complete, $\frac{1}{6}$ P and $\frac{1}{30}$ P; (*b*) *Nitrogen*

experiment; and (*c*) *Potassium experiment* with three corresponding levels o nitrogen and potassium respectively. Except in the phosphorus experimen the nutrient solutions applied were of the same strength as in the barley experiments (Humphries, 1951). No renewal of nutrient solution was made during the growing period, and as in the case of the barley experiment a duplicate set of tanks was set up exactly a week later. Four days prior to sampling, half the tanks in each treatment were darkened by covering with light-tight shades to provide plants with a lower carbohydrate content.

The plants were sampled about 5 weeks after germination when most o them had produced one flower. The procedure followed the same lines as with the barley, and samples of excised roots were treated with a complete aerated nutrient solution for 2, 4, 8, 16, and 24 hours at 30° C. An initial sample (0 hr.) was also taken. In the phosphorus experiment samples for carbohydrate analyses were taken at all the sampling times, but in the other 2 experiments at 0, 8, and 16 hours only.

Respiration rates were determined on the complete and lowest level o phosphorus in the phosphorus experiment and on the two deficient levels o nitrogen and potassium in the nitrogen and potassium experiments respectively. The analytical procedures were as previously mentioned (Humphries loc. cit.) except that sucrose and reducing sugars were determined on extracts cleared with basic lead acetate (Humphries, 1943) to increase the speed o analysis.

EXPERIMENTAL RESULTS

The effect of cultural treatment on yield of tops and roots

A. *Phosphorus experiment.* In Table I are presented the fresh weights and dry weights of the tops and the fresh weights of the excised roots before treatment. Each weight was the mean of 12 samples. It will be seen that th

TABLE I

Yield of tops and roots of pea plants grown in water culture

Time of year plants were grown	Fresh weight (g.) per 18 plants				Dry weight (g.) per 18 plants	
	Roots		Tops		Tops	
	Unshaded	Shaded	Unshaded	Shaded	Unshaded	Shaded
Complete May–June	43·4	40·2	137·0	122·0	12·8	10·1
$\frac{1}{6}$ P	49·6	45·9	117·3	100·2	12·5	9·3
$\frac{1}{30}$ P	48·1	47·8	89·5	76·2	10·4	8·1
L.S.D.	3·39		6·50		0·76	
Complete June–July	22·4	23·9	63·2	63·0	6·7	6·0
$\frac{1}{6}$ N	30·4	30·3	56·7	55·1	6·8	5·5
$\frac{1}{30}$ N	32·3	32·0	49·1	43·3	6·1	4·5
L.S.D.	3·45		8·18		0·77	
Complete Aug.–Sept.	27·8	28·0	106·0	100·5	12·2	10·1
$\frac{1}{6}$ K	23·3	21·0	85·1	78·3	10·6	8·5
$\frac{1}{30}$ K	24·1	21·6	72·1	67·0	9·4	7·7
L.S.D.	3·28		6·69		0·82	

effect of 4 days' shading was to reduce the fresh weight of both roots and tops; the dry weight of the tops was also reduced. The roots of the plants grown in the phosphorus-deficient solutions had higher fresh weights than those grown in complete solution—the well-known effect of phosphorus deficiency in producing a larger root system. On the other hand, increasing phosphorus deficiency decreased the fresh weight and dry weight of the tops.

B. *Nitrogen experiment.* The roots of the complete treatment had a lower fresh weight than the roots of the two levels of nitrogen deficiency. Shading did not decrease the fresh weight of roots in any of the treatments. The effect of increasing nitrogen deficiency, however, is apparent on the fresh weight of tops. Shading also tended to decrease the fresh weight of tops, especially in the $\frac{1}{30}$ N treatment. The dry weight of the tops was very similar in all treatments, but shading decreased the dry weight. It will be observed from Table I that the fresh weights of the complete treatment of the N experiment were only about half that of the phosphorus experiment, and this is because the plants experienced somewhat adverse growth conditions due to a very hot spell when apparently the aeration of the culture solution was not adequate; no forced aeration was employed in these experiments.

C. *Potassium experiment.* The effect of potassium deficiency was to decrease the fresh weight of the roots a little. Shading also had only a small effect. The fresh weight of the tops was decreased much more by potassium deficiency than that of the roots.

In all these experiments, with the possible exception of the phosphorus experiment, the magnitude of the effect on the fresh and dry weights of the deficiencies present in the culture solutions was a good deal less than would have been expected and certainly less than those experienced in the corresponding barley experiments. Probably a part of the explanation of this is to be found in the size of the seed reserve of peas compared with that of barley, and as the plants were only grown for a few weeks no marked deficiencies were induced because of the mineral supply of the cotyledons. In spite of this, however, the content of element in the deficient plants was much smaller than the content of that element in the complete treatments, as will be seen later, so in this sense they may be considered to be deficient plants although this is not always apparent from the yield of root material.

The effect on fresh weight of excised roots when exposed to an aerated complete culture solution

There was a change of fresh weight due to changes in both dry matter and water content during the treatment of the excised roots with an aerated culture solution, as was found in the barley experiments. The relative parts played by water content and dry matter in contributing to changes in fresh weight during treatment has been estimated in the manner that was employed with the barley data. The final fresh weight has been expressed as a percentage of the fresh weight before treatment, and from this has been subtracted the

dry matter expressed as a percentage of fresh weight before treatment. This gives the water content as a percentage of the value before treatment.

The changes in water content and dry matter in the three treatments o

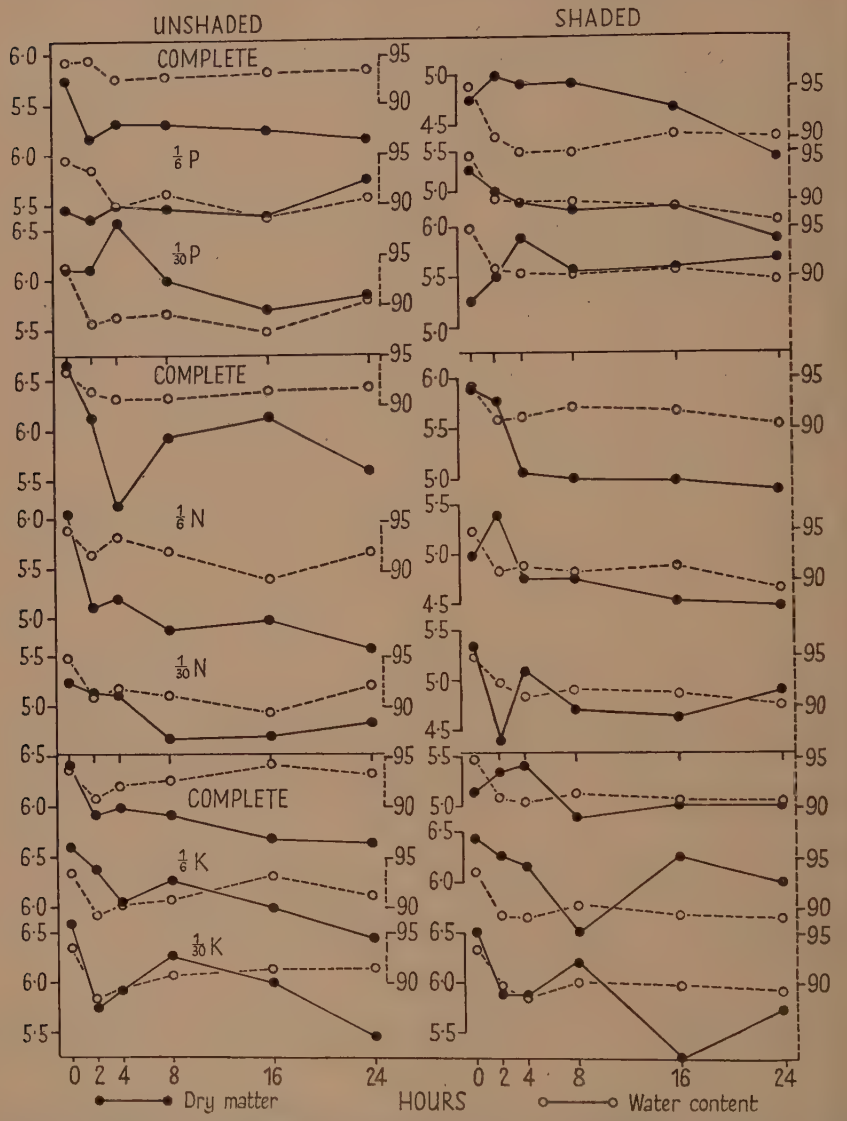

FIG. 1. The changes in dry matter and water content of excised roots of pea plants grow under varying nutrient conditions during the course of 24 hours. Ordinates on the left sho dry matter and on the right show water content as percentage of the original fresh weigh

each of the three experiments are shown in Fig. 1. In general, there is progressive loss of dry matter during the 24 hours' treatment and this loss often greatest in the first few hours. There are several instances of an increa

in dry matter above the initial value. As there is no means whereby the excised roots could increase appreciably in dry matter, this is just an expression of the rather large variability of the material—much greater than found in the barley plants—and this presumably must be attributed to variation in the cotyledon reserves of the pea plants.

In most of the experiments there was also a progressive decrease in water content during treatment. This was invariably the case with the shaded samples, but in the potassium experiment, roots from the unshaded treatments tended to gain water after an initial fall. In the barley experiments there was an indication that both sugar content and rate of potassium uptake influenced the rate of water uptake. The data for the excised pea roots have been analysed in a similar manner, i.e. quadratic regressions were fitted to the changes in water content with time and from these the rate of water uptake or loss calculated at 0 hours, 8 hours, and 16 hours. The partial regressions of this rate on the appropriate sugar content and rate of potassium uptake (see p. 303) were calculated.

TABLE II

Partial regression coefficients of rate of water uptake on rate of potassium uptake (pooled data)

Those significant marked with asterisk

	Partial regression coefficient
Phosphorus experiment . . .	$+0{\cdot}017^{*}\pm0{\cdot}008$
Nitrogen experiment . . .	$+0{\cdot}021^{*}\pm0{\cdot}005$
Potassium experiment . . .	$+0{\cdot}004\ \pm0{\cdot}008$

In three instances the rate of water uptake was correlated with sugar content, but no instance of correlation with potassium uptake was evident. If the data for 0 hours, 8 hours, and 16 hours in each experiment are pooled, the range of rates of potassium uptake over which the regressions are calculated is much greater, and there is then an indication of correlation of water uptake with rate of potassium uptake in the phosphorus and nitrogen experiments. The partial regressions of rate of water uptake on rate of potassium uptake for the pooled data are shown in Table II. There seems to be some evidence from the experiments with peas, as with those with barley, that uptake of water is directly related to uptake of potassium, but it will be necessary to design specific experiments to test this before a more definite conclusion can be reached.

Changes in phosphorus, nitrogen, and potassium of the excised roots

The changes in phosphorus, nitrogen, and potassium expressed as a percentage of the initial fresh weight have been plotted in Figs. 2, 3, and 4 and smoothed curves have been drawn through the points; these were calculated by the method already adopted for the barley data (Humphries, loc. cit.).

A. *Phosphorus experiment.* The changes in phosphorus content of the excised roots are shown in Fig. 2. The roots of unshaded plants grown at all three

levels of phosphorus gained in phosphorus during the 24-hour period o
treatment. The roots of shaded plants of the complete treatment lost a sma

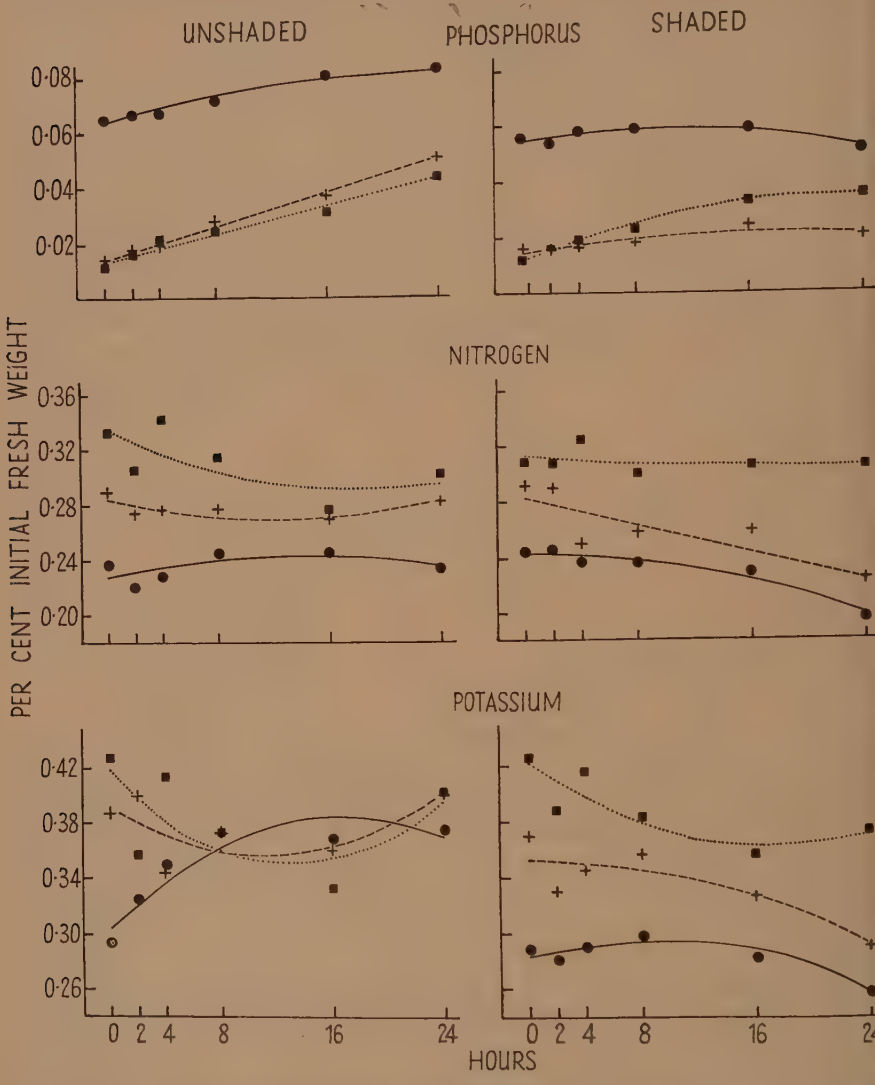

FIG. 2. The changes in phosphorus, nitrogen, and potassium content of excised roots of p
plants grown under varying conditions of *phosphorus* supply, when subjected to an aera
complete culture solution.

●—— complete, + - - - - $\frac{1}{6}$ P, ■ . . . . $\frac{1}{30}$ P.

amount of phosphorus, but the roots from the two deficient levels of phospho
gained in phosphorus, but not as rapidly as the roots of the unshaded plan

There was a slight gain of nitrogen by the roots of unshaded plants of t
complete series, while the roots from the other two treatments showed a sli

loss of nitrogen, and there was a progressive increase in nitrogen content of the roots with decrease in phosphorus content of the nutrient solution in

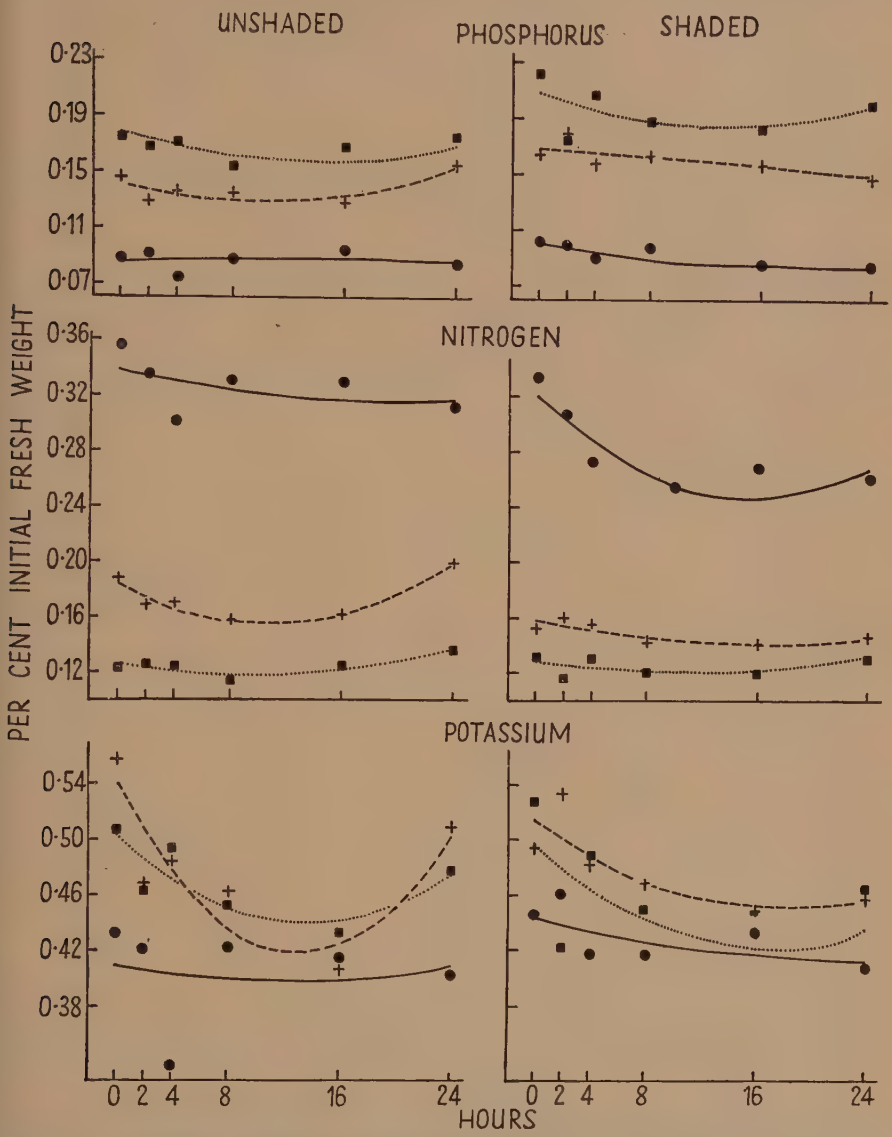

FIG. 3. The changes in phosphorus, nitrogen, and potassium content of excised roots of pea plants grown under varying conditions of *nitrogen* supply, when subjected to an aerated complete culture solution.

●—— complete, +– – – – $\frac{1}{6}$ N, ■. . . . $\frac{1}{30}$ N.

which they were grown. Roots of the complete series had the lowest nitrogen content. Roots from shaded plants grown at all three phosphorus levels

showed a decrease in nitrogen content with time. The potassium content of roots from the unshaded complete treatment showed an increase during the first 12 hours, while in the case of the other two treatments the potassium content declined with a tendency to increase again at the end of the period. There was a loss of potassium from the roots of shaded plants in the $\frac{1}{6}$ P and $\frac{1}{30}$ P treatments and very little change in the potassium content of the roots of plants grown in complete solution.

B. *Nitrogen experiment.* The phosphorus content of roots of the complete series showed little change with time (Fig. 3), while the roots of the other treatments showed slight losses. The roots of the shaded plants of all three series lost phosphorus. The nitrogen content of the roots with the complete treatment declined, while there was little change in the nitrogen content when $\frac{1}{6}$ N and $\frac{1}{30}$ N treatments were given. There was a big decline in nitrogen content of the complete shaded plants and small decreases with the other two treatments. The potassium content with all treatments, both shaded and unshaded, decreased, but there was a tendency in some treatments to increase again towards the end of the period.

C. *Potassium experiment.* There was very little change in the phosphorus content of the roots with all three treatments either shaded or unshaded (see Fig. 4). The nitrogen content with the complete and $\frac{1}{6}$ K unshaded treatments declined in N content while the $\frac{1}{30}$ K treatment increased. All the shaded samples decreased in nitrogen content. The potassium content of the complete unshaded series decreased, while in the other two treatments ($\frac{1}{6}$ K and $\frac{1}{30}$ K) it increased. The only treatment of the roots of shaded plants which gave an increase in potassium content was the $\frac{1}{6}$ K treatment.

Conclusion. The excised roots of peas behaved similarly to those of barley in that there were numerous examples of simultaneous gain of one ion and a loss of another, e.g. in the phosphorus experiment the roots of the $\frac{1}{30}$ P unshaded treatment gained phosphorus and at the same time lost nitrogen and potassium; in the $\frac{1}{6}$ K treatment (unshaded) the roots lost nitrogen at the same time that they gained in potassium.

Changes in carbohydrate content of the excised roots

Estimation of soluble carbohydrate (sucrose and total reducing sugars) and of starch were made only on certain samples—in the case of the phosphorus experiment at all six sampling times and in the other two experiments at 0, 8, and 16 hours only. The amount of starch found was extremely small and showed little change with time, and starch content has been neglected in considering the effect of carbohydrate content on uptake.

The changes in sucrose and reducing sugars of the excised roots during treatment with complete culture solution is shown in Table III. The figures call for little comment; they show that there was a rapid diminution in sucrose content in the first few hours falling to a very small value after 24 hours. The effect of shading was to decrease the initial sugar content considerably. The

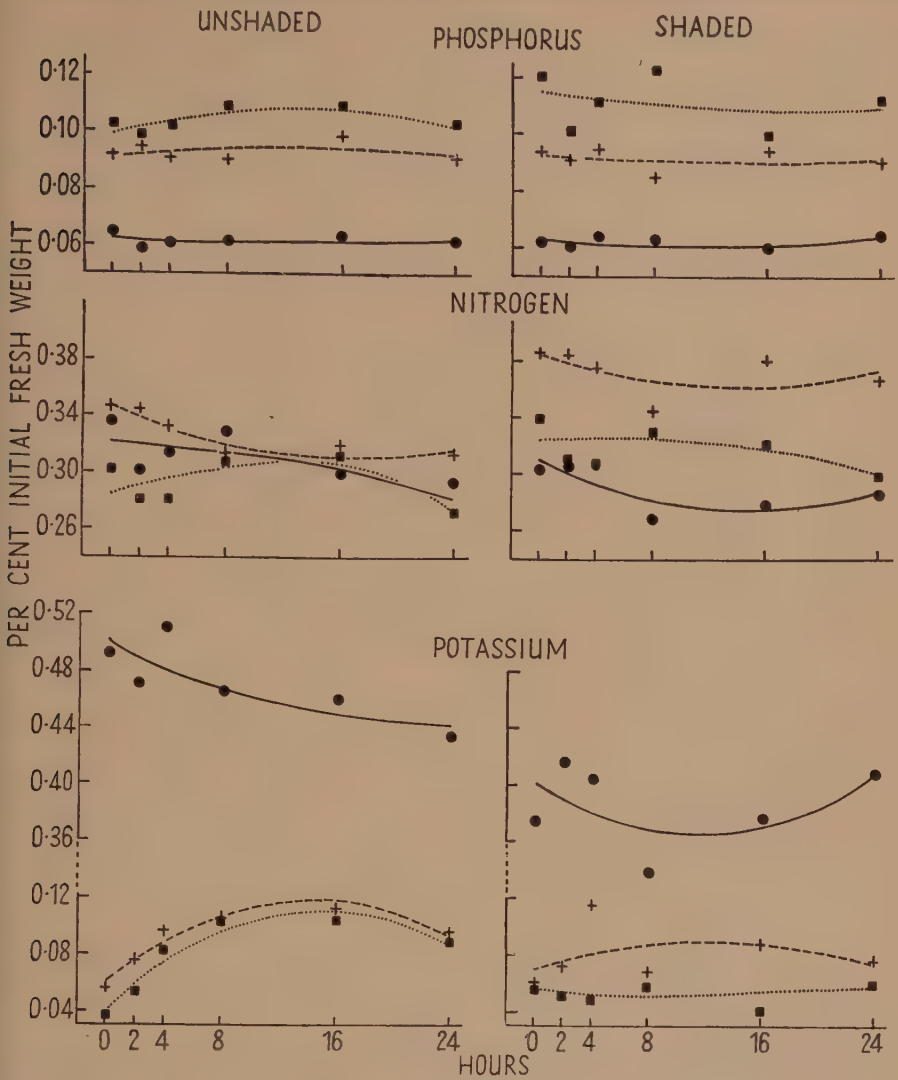

FIG. 4. The changes in phosphorus, nitrogen, and potassium content of excised roots of pea plants grown under varying conditions of *potassium* supply, when subjected to an aerated complete culture solution.

●—— complete, + – – – – $\frac{1}{6}$ K, ■ . . . . $\frac{1}{30}$ K.

reducing sugar content of roots of unshaded plants grown at the two levels of nitrogen deficiency was higher than that of the roots grown in complete nutrient.

Changes in respiration rate of the excised roots

The respiration rate of certain samples of the excised roots over a 16-hour period of immersion in aerated flowing complete culture solution at constant

Table III

Sucrose and reducing sugar content of excised roots (as a percentage of initial fresh weight) of pea plants grown under conditions of varied nutrient supply and subjected to a complete aerated culture solution for different periods of time

	Complete nutrient				$\frac{1}{6}$ phosphorus				$\frac{1}{30}$ phosphorus			
	Unshaded		Shaded		Unshaded		Shaded		Unshaded		Shaded	
Hrs.	Sucrose	R.S.	Sucrose	R.S.	Sucrose	R.S.	Sucrose	R.S.	Sucrose	R.S.	Sucrose	R.S.
0	0·438	0·108	0·015	0·022	0·341	0·026	0·054	0·020	0·370	0·021	0·073	0·015
2	0·217	0·073	0·016	0·026	0·161	0·022	0·019	0·016	0·191	0·015	0·039	0·041
4	0·150	0·039	0·011	0·015	0·161	0·013	0·018	0·023	0·270	0·012	0·043	0·016
8	0·279	0·039	0·003	0·015	0·135	0·013	0·008	0·013	0·114	0·012	0·019	0·019
16	0·046	0·011	0·002	0·016	0·059	0·012	0·003	0·013	0·064	0·010	0·002	0·010
24	0·039	0·010	0·004	0·016	0·014	0·026	0·004	0·007	0·020	0·014	0·008	0·013
	Complete nutrient				$\frac{1}{6}$ nitrogen				$\frac{1}{30}$ nitrogen			
0	0·281	0·029	0·012	0·055	0·163	0·180	0·014	0·050	0·056	0·186	0·009	0·062
8	0·085	0·021	0·004	0·019	0·109	0·049	0·004	0·066	0·097	0·184	0·011	0·048
16	0·019	0·036	0·004	0·014	0·009	0·039	0·003	0·021	0·028	0·077	0·004	0·029
	Complete nutrient				$\frac{1}{6}$ potassium				$\frac{1}{30}$ potassium			
0	0·200	0·028	0·037	0·002	0·169	0·121	0·063	0·011	0·039	0·053	0·108	0·021
8	0·073	0·006	0·011	0·013	0·104	0·008	0·029	0·020	0·069	0·021	0·053	0·010
16	0·023	0·006	0·042	0·011	0·016	0·020	0·006	0·019	0·030	0·004	0·005	0·013

temperature was measured by the same method as previously described for barley roots. In the phosphorus experiment the respiration rate of the complete and $\frac{1}{30}$ P samples was measured, but in the other two experiments the respiration rates of samples from the two deficient levels were recorded. The results are presented graphically in Fig. 5. In all samples there was a steady decline in

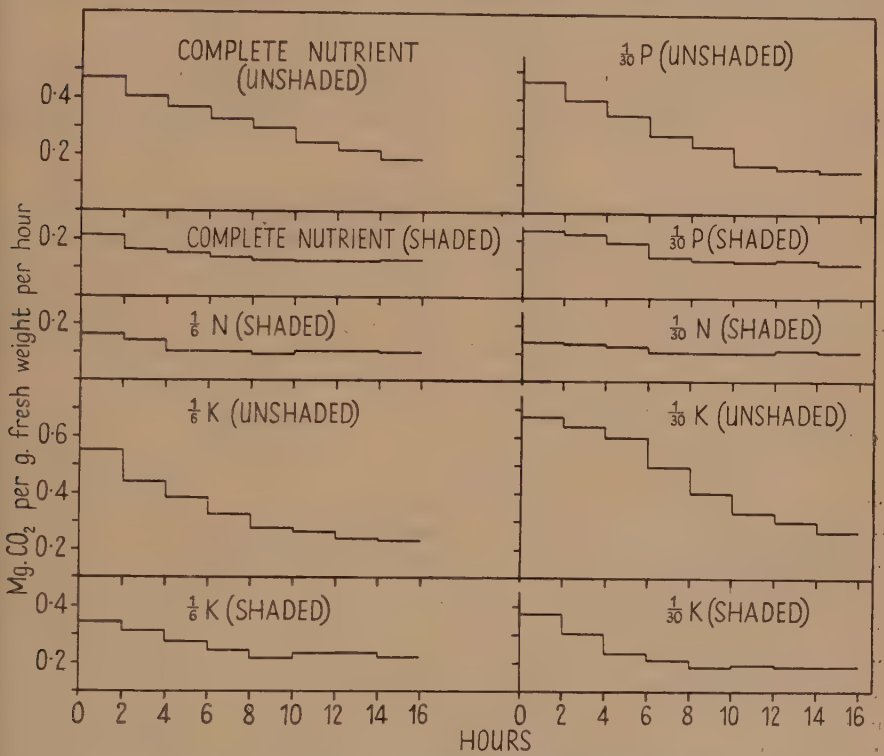

FIG. 5. The respiration rate of excised roots of pea plants, grown under varying conditions of nutrient supply, in a complete aerated culture solution.

respiration rate during the 16 hours. The initial rate of the unshaded samples was always higher than that of the corresponding shaded samples and fell more steeply with time than did the latter. There was a failure of the respiration apparatus during the measurement of the respiration rate of $\frac{1}{6}$ N samples, so that no results for this treatment have been shown in the diagram. In none of the experiments was there any marked increase in N content of the samples, and corresponding with this there was no increase in respiration rate. In roots in which either phosphorus or potassium was absorbed, no increase in respiration rate ('salt respiration') was observed in conformity with the observations on barley roots.

The respiration rate of excised barley roots was found (Humphries, loc. cit.) to depend both on sucrose content and on nitrogen content, and the present data have been analysed to see if a similar relationship held with pea

roots. In the phosphorus experiment sucrose estimations were made at all sampling times, so that more data are available in this experiment than in the subsequent ones. This experiment was therefore analysed by finding the partial regressions of respiration rate on (*a*) total nitrogen, (*b*) sucrose, and (*c*) reducing sugars at each nutrient level, unshaded and shaded samples being treated separately. The results are shown in Table IV. In no instance was the

TABLE IV

Partial regression coefficients of respiration rate on nitrogen content and sucrose content

(g. CO_2 per hr. per g. nitrogen or sucrose) × 10.
Those significant marked with an asterisk.

		Nitrogen content	Sucrose content
Phosphorus expt.	Complete unshaded . .	−1·1 ±2·25	0·7*±0·29
	,, shaded . .	5·5*±1·86	−0·1 ±1·69
	$\frac{1}{30}$ P unshaded . .	2·4*±1·13	0·9*±0·20
	,, shaded . .	−0·1 ±1·35	1·7*±0·43
Potassium expt.	All treatments 0 hr. .	−1·8*±0·60	1·1*±0·21
	,, ,, 16 ,, .	0·5 ±0·41	5·1*±1·66

partial regression of respiration rate on reducing sugar content significant and the values obtained have not been tabulated. In two cases in the phosphorus experiment, viz. the complete shaded and $\frac{1}{30}$ P unshaded, the partial regressions of respiration rate on nitrogen content are significant, and in these two instances there is a loss of N during the course of the experiment. In the other two examples there was either a slight rise or practically no change in N content. A feature of the experiments with peas is that no considerable losses of nitrogen from the excised roots took place, which is in marked contrast to the experience with barley. In the potassium experiment all treatments have been considered separately at 0 and 16 hours. Here the partial regression on N is significant at 0 hours (negative) but not at 16 hours. The partial regressions on sucrose are significant on both occasions.

In the barley experiments a comparison of the amount of sugar lost with the amount of carbon dioxide evolved over a 16-hour period showed that, except in three instances, the carbon dioxide evolved in respiration is greatly in excess of the carbon dioxide equivalent of the sugar consumed during the same period. Two out of three of these exceptions occurred in samples in which protein synthesis was occurring. A comparison of the carbon dioxide evolved and carbon dioxide equivalent of the sugar consumed during an 8-hour and a 16-hour period by the excised roots of peas is shown in Table V. In only two samples does the carbon dioxide equivalent of the sugar consumed exceed the carbon dioxide evolved, viz. the complete unshaded and $\frac{1}{30}$ P unshaded samples of the phosphorus experiment, but in neither instance is there any evidence of actual protein synthesis.

TABLE V

CO_2 evolved in respiration and CO_2 equivalent of sugar consumed during 8-hour and 16-hour periods

(mg. per 100 g. fresh weight)

	Treatment	8-hour period		16-hour period	
		CO_2 evolved	CO_2 equivalent of sugar consumed	CO_2 evolved	CO_2 equivalent of sugar consumed
Phosphorus expt.	Complete unshaded	315	347	504	748
	Complete shaded	135	29	238	29
	$\frac{1}{30}$ P unshaded	297	408	439	489
	$\frac{1}{30}$ P shaded	158	83	260	117
Nitrogen expt.	$\frac{1}{8}$ N shaded	103	15	186	60
	$\frac{1}{30}$ N shaded	101	21	187	56
Potassium expt.	$\frac{1}{8}$ K unshaded	338	266	540	384
	$\frac{1}{8}$ K shaded	235	53	416	88
	$\frac{1}{30}$ K unshaded	477	47	746	86
	$\frac{1}{30}$ K shaded	223	101	377	171

Factors controlling loss or gain of ions

Similar experiments to those described in this paper which had previously been performed on excised roots of barley established that two factors determining gain or loss of a particular element are the concentration of that element present in the root cells and the carbohydrate content of the root. When the content of an element is low, the roots tend to increase in content of that element, while roots with a high content of the given element tend to lose it. High-carbohydrate roots are capable of absorbing the element, while exosmosis of the element occurs in low-carbohydrate roots. This relationship was established by first calculating the rates of uptake of the three elements, phosphorus, nitrogen, and potassium, from the quadratic regression equations of content of element on time. The rates of uptake of a particular element were then related to the content of that element and the sugar content, by calculation of partial regression of rate on these two variates.

This procedure has been followed with the present data, but in addition to rates at 0 hours and 16 hours the rates at 8 hours could be calculated, as carbohydrate data were also available at that time. The data from excised pea roots were found to be much more variable than the corresponding data from barley roots, and this is probably largely due to the much more variable reserve of both mineral substances and carbohydrate in the pea cotyledons. This greater variability resulted in fewer significant partial regression coefficients, but the same general relationship between rate of uptake, content of element, and carbohydrate content held good.

The partial regression coefficients of rate of uptake of nutrient (N, P, or K) on content of nutrient and on sugar content at 0, 8, and 16 hours are set out in Table VI. The total variance of uptake and the percentage of the variance

Table VI

Partial regression coefficients ($\times 10^3$) of rate of uptake (mg./100 g./hr.) of nitrogen, phosphorus, or potassium on content of nitrogen, phosphorus, or potassium and on total sugar content, initially and after 8 and 16 hours treatment with complete aerated culture solution.

V = total variance of rate of nutrient uptake on nutrient content (mg./100 g. fresh wt./hr.) $\times 10^6$.
$V\%$ = per cent. of variance accounted for by regression.
b_1 = regression of rate of uptake of nutrient (N, P, or K) on content of that nutrient.
b_2 = regression of rate of uptake of nutrient (N, P, or K) on total sugar content.
* Indicates a significant regression coefficient.

	Nitrogen			Phosphorus			Potassium		
initial	N expt.	P expt.	K expt.	N expt.	P expt.	K expt.	N expt.	P expt.	K expt.
b_1	−22·8*	−59·6*	−20·2	−19·6	−6·4	−20·5	−107·1*	−94·8*	−21·5*
	±10·81	±18·15	±24·04	±14·75	±8·07	±15·12	±41·46	±18·44	±5·09
b_2	4·1	1·8	8·2	−0·3	1·2	4·9*	4·5	4·7	21·6*
	±6·79	±2·95	±9·06	±4·27	±0·83	±2·42	±12·56	±5·65	±6·87
V	188·8	104·1	192·3	56·0	4·3	17·1	692·2	692·6	452·5
$V\%$	20	45	0	0	2	21	30	70	76
8 hours									
b_1	−7·9	−15·6	21·0	−6·9	−15·6*	−3·3	0·4	−59·5*	−14·2*
	±6·16	±12·43	±12·84	±5·80	±7·40	±8·18	±25·16	±18·15	±4·30
b_2	6·7	5·3*	−40·5*	0·7	2·3*	3·3	0·5	19·7*	−15·1
	±4·74	±2·61	±19·82	±2·19	±1·09	±6·13	±9·24	±4·80	±21·77
V	37·1	23·3	25·5	6·4	3·2	3·3	98·5	139·1	92·2
$V\%$	25	21	17	0	26	0	0	62	46
16 hours									
b_1	−7·1	24·4	−2·3	−0·1	−10·9	−5·2	−19·6	−7·5	−1·3
	±6·28	±12·73	±11·98	±2·77	±11·72	±3·17	±16·45	±27·10	±2·60
b_2	−4·1	20·0	8·1	2·6	8·7	−2·8	16·7	21·5	54·6*
	±13·33	±10·46	±26·68	±9·82	±6·67	±3·49	±23·36	±27·51	±23·76
V	30·6	26·1	23·1	14·9	7·1	0·5	94·1	96·4	25·5
$V\%$	0	35	0	0	4	7	0	0	24

accounted for by the regression are also tabulated. It will be seen that, as in the case of the barley data, the greatest variance is at 0 hours and is much diminished at 8 hours and 16 hours. The values of the variance were greatest for potassium in all three experiments. Considering the table as a whole it is evident that when the total variance is large the partial regression coefficients are usually significant, suggesting that failure to attain significance may be due to the small range of values over which the regressions are calculated.

The data were also examined to see if there was any clear evidence of rate of uptake of one element being influenced by another. The procedure adopted was the same as in the barley experiments, i.e. an independent variate (in this case the content of one of the elements not already taken into account; either phosphorus, nitrogen, or potassium) was added to the multiple linear regression and its significance estimated by the procedure due to Cochran (1938). In all 54 additional partial regression coefficients were calculated (9 coefficients for P rate on P content, sugar content, and N content; 9 for P rate on P content, sugar content, and K content, &c.). Eleven coefficients proved to be significant; three of these for the rate of N uptake taking K content into account, and three for the rate of K uptake taking P content into account. These results are rather inconclusive, but do not suggest that the rate of uptake of the three elements are dependent on one another. A similar conclusion was reached with the barley data.

In the analysis presented, partial linear regressions have been fitted to the data so that the regression of rate on sugar content implies that the rate of uptake steadily increases with increasing sugar content. It is possible, however, that there is a limiting sugar content above which no further increase in rate occurs. This possibility was examined by fitting a quadratic regression involving the square of the sugar content. This procedure was tested in experiments where the range of sugar content was large. Certain of the barley experiments were tested in the same way. In no case was the quadratic term significant and invariably the curvature increased with increasing sugar content. It was therefore concluded that there was no evidence at all of a limiting sugar concentration over the range encountered in these experiments.

DISCUSSION

The experiments with excised pea roots, allowing for the greater variability of the material, have served to confirm the conclusions previously reached in experiments with excised barley roots, that uptake of any element is controlled both by the sugar content and by the content of that element.

There is a growing volume of literature on the effect of sugar (and the correlated effect of light) on ion uptake, and a recent paper by Van Andel, Arisz and Helder (1950) includes some references to this subject. These authors found, using a modification of the apparatus of Woodford and Gregory (1948), that with intact maize plants in the dark the uptake of phosphate decreased but that uptake of phosphate was enhanced in the presence of

glucose in the dark. Also with plants continuously exposed to light, glucos enhanced uptake. When the plant, after a dark period with glucose, was onc more placed in the light but without glucose, the uptake of phosphat diminished. They found that the effect of light and glucose on uptake c potassium was essentially the same as phosphate. These conclusions are quit in accord with the evidence presented in this and the previous paper.

An implicit deduction from the data is that when sugar concentration fall below a certain value exosmosis of ions is likely to occur. As yet the exact rol of sugar in this connexion can only be surmised. It may have a direct effect a a source of energy necessary to transport the ions against a gradient, but i addition it may be the parent substance for the formation of a chemica compound capable of combining with ions. This theory was put forward b Jacobsen and Overstreet (1947). They postulate that absorbed ions are fixed i the cells in the form of chemical compounds. They suggest that since io absorption is dependent upon oxygen consumption, it is conceivable that thes binding substances are produced during metabolism, either directly as inter mediates of aerobic respiration or indirectly as products of metabolic syn thesis. The dependence of rate of uptake of ions on sugar concentration fit in with this hypothesis. Sugar, either by respiratory breakdown, or as starting-point for more elaborate synthesis, could conceivably give rise to substance which acts as the ion-binding compound. The present work suggest that both anions (nitrate and phosphate) and cations (potassium) obey th same laws of absorption (dependence of rate of uptake on sugar concentratio and on concentration of element in the cells) and the hypothetical ion-bindin substances must be capable of combining with either anions and cations. It i extremely improbable that a single substance exists which could combin with all the species of cations and anions which normally accumulate in plan cells and it is far more likely that a specific substance is responsible for bindin a particular ion. Cowie *et al.* (1949) and Roberts *et al.* (1949) studied potassiun metabolism in *Escherichia coli* and inferred from their results that the cel contain a potassium binding compound (or closely related compounds). N binding of sodium was observed. The addition of glucose to the mediun caused a large uptake of potassium. Similar experiments with a chlorophyl containing organism, *Chlamydomonas humicola*, showed increased uptake c potassium when illuminated. Most of their results were in agreement with th hypothesis that potassium is bound as the di-potassium salt of the hexose phosphates. They conclude: 'The phenomena observed with *E. coli* seem ver similar to those observed in other organisms and tissues. It seems that th type of binding may be fairly general and in conjunction with membranes c low permeability could explain most of the observations without any require ment for "active transport" by membranes.'

Substances present in cells capable of binding phosphate ions, such a hexoses, are already well known, and ammonium and nitrate ions are readil incorporated into higher nitrogenous compounds by the process of protei synthesis. On these assumptions, therefore, the cation potassium and the tw

anions nitrate and phosphate would be dependent on the carbohydrate status of the cell for the formation of substances capable of binding them. This would readily explain the dependence of uptake of these three ions on carbohydrate content. It may be that the accumulation by plant cells of ions which are of little or no importance in metabolism, such as chloride and bromide, takes place by a different mechanism and perhaps here 'active transport' is necessary for their accumulation. If the hypothesis of 'ion binding' substances is to prevail it will be necessary to discover compounds which are capable of binding such ions as Na which accumulate and are apparently used in metabolism in certain groups of plants.

The conclusion that rate of uptake of a given element depends on the concentration of that element in the cell—the rate of uptake being greater at low concentration of element—also supports the ion-binding hypothesis. It would be expected that if an ion is combining with a molecule inside the cell, the rate would be greatest when there are the greatest number of points of attachment available and as those become progressively occupied the rate would decline and finally reach zero when the ion-binding substance is fully saturated.

Any theory must take into account the phenomenon of simultaneous loss or gain of ions as indicated by the present series of experiments.

The present experiments with excised peas, like those with barley roots, failed to show any increase in respiration rate when accumulation of ions was taking place. Robertson (1951) states 'not all tissues show an increase in respiration on addition of salt; thus roots from a culture solution or storage tissues just cut require some time in aerated distilled water before an increase is observed on addition of salt. Such tissues also increase in capacity for absorption.'

It has been demonstrated, however (Humphries, 1950, 1951), that roots from a culture solution may be capable of considerable accumulation of ions, while at the same time the respiration rate is falling. The question therefore arises whether the mechanism of accumulation in such roots is entirely different from the mechanism postulated by Lundegårdh and Robertson, and in support of which a good deal of evidence has been adduced, or whether the same mechanism operates, but that the visible manifestation of it, increased respiration rate, is masked by other metabolic processes which result in a declining respiration rate. This is a possible explanation, since Robertson (1941) has shown that under the conditions of his experiments the energy liberation represented by the extra CO_2 evolved is at least 100 times greater than the energy necessary to account for the observed accumulation. It may be that under other conditions such as accumulation by freshly cut excised roots, the energy necessary for accumulation and the amount of CO_2 evolved more closely correspond. If so, the CO_2 evolved as a result of work done during accumulation would be negligible compared with the CO_2 arising from the normal metabolic processes and would not be distinguished from this.

On the other hand, the fact that tissues have to be preconditioned (disks

washed in aerated water) for a considerable time before accumulation asso ated with a rising respiration rate is observed rather suggests that anoth mechanism, e.g. the cytochrome system, present in the cell all the time becom much more active and the phenomenon of 'salt respiration' results. In th connexion the observations of Chin (1950) are of interest. He found that aeration of brewers' yeast parallel changes in both the cytochrome co ponents and the respiratory activity could be induced. After 8 hours' aerati the oxygen uptake of brewers' yeast increased by 60 per cent. and at the sam time a distinct change had occurred in appearance of the spectrum of t cytochrome components. He concluded that apparently the formation of ne cytochrome components takes place at the expense of other components the same system. It is possible that somewhat similar changes take place duri the aeration of carrot disks and may explain why it is necessary to preconditi such tissues before the phenomenon of salt respiration is evident. It therefo seems to be an open question whether the theory of 'anion' or 'salt' respirati applies to the normal process of accumulation by roots. It is possible th uptake due to the activity of the cytochrome system is a phenomenon whi only occurs under particular circumstances. There is also the possibility th different kinds of tissues have different mechanisms for absorption of ion Österlind (1951) has shown that the respiration of *Scenedesmus quadricau* is quite unaffected by a wide range of cyanide concentrations whereas t absorption of nitrate is totally inhibited by low concentrations. The theory anion respiration seems to be completely ruled out in this instance.

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The Use of Sodium Diethyldithiocarbamate as a Respiratory Inhibitor

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SUMMARY

A technique for the application of dieca (diethyldithiocarbamate) to plant tissues is described. It is shown to differentiate almost quantitatively between extracted ascorbic oxidase and polyphenolase on the one hand and cytochrome oxidase on the other at a concentration of M/5,000. It produces strong, but not total, inhibitions when applied to barley root-tips.

INTRODUCTION

One of the most urgent requirements in the present investigation of respiratory oxidations in plants is a non-competitive inhibitor of copper systems that will not react appreciably with iron. A number of organic compounds are now available that have a high degree of specificity for copper and which react at low concentrations, M/1,000 to M/10,000, under mild conditions. Preliminary tests eliminated most of them as unsuitable for the present purpose. Of those listed by Albert and Gledhill (1947) as reacting more or less specifically with copper at pH 7 and 37° C., benzoinoxime and thiourea were useless because they were too insoluble in biological media.

Potassium ethylxanthate is given by Albert and Gledhill as reacting with Cu^{++} and not with ferrous or ferric ions. It has also been employed as an inhibitor of ascorbic oxidase from cauliflower, squash, and cabbage (Kitagawa, 1939). We investigated a commercial sample and four separate preparations made from analar reagents by Dr. B. H. Thewlis, using the method of Rast (1910). The last preparation was recrystallized from alcohol immediately before use. At a concentration of M/1,000 it precipitated M/10,000 $CuSO_4$ at room temperature and approximately pH 7. Ferrous sulphate was not precipitated under these conditions.

The ascorbic oxidase prepared from young barley leaves as under was totally inhibited at M/1,000. Despite the negative results with ferrous sulphate, cytochrome oxidase, prepared by Goddard's (1944) method, was inhibited 66 per cent. at M/1,000 and 39·6 per cent. at M/2,500. No material differences were obvious between samples. Potassium ethylxanthate, therefore, in spite of its failure to react with iron offered little prospect of differentiating satisfactorily between copper systems and cytochrome oxidase.

A number of attempts have been made to use sodium diethyldithiocarbamate ('dieca'). The principal difficulty is that it is unstable, especially in mildly acid solutions. During decomposition in manometers, it gives rise to a positive gas pressure, which might be mistaken for an inhibition.

O_2-consumption. Realization of its instability has caused some investigators to abandon the attempt to use it, but in the absence of visible alternatives it seemed worthy of further investigation.

MATERIALS AND METHODS

Sodium diethyldithiocarbamate. B.D.H. analar grade was used throughout and dissolved in glass-distilled water or M/15 phosphate buffer immediately before use. It was usually made up at M/100 and M/500 for dilution to M/1,000 and M/5,000.

Ascorbic acid. B.P. grade was dissolved and neutralized in glass-distilled water or buffer immediately before use.

Ascorbic oxidase. Preparations were made from etiolated leaves of young barley seedlings as described by James and Cragg (1943). The solutions were quite clear, and showed no spontaneous uptake of oxygen before addition of ascorbic acid.

Cytochrome oxidase was prepared by Goddard's method with acetate precipitation; *p*-phenylenediamine or ascorbic acid was not oxidized, or oxidized only very slowly, before addition of cytochrome *c*.

p-Phenylenediamine, Kahlbaum, was dissolved immediately before use and supplied at the rate of 10 mg. per flask.

Cytochrome c Evans. A sterile preparation as used for intravenous injection and containing an assayed quantity of 50 mg. per ml.

M/15 phosphate buffers were made up in glass-distilled water, and checked with the glass electrode or Hellige comparator, and adjusted to within 0·1 unit.

Oxygen consumption was measured in Warburg manometers in single side-arm flasks with 10 per cent. KOH saturating Whatman No. 1 filter-paper in the central wells. Flasks were equilibrated to bath temperature by shaking for 25 minutes or longer with open taps. Experiments were run with duplicate flasks for each treatment, and occasional failures due to detectable causes were rejected.

Root-tips. Barley, variety 'Spratt Archer' of the latest harvest, was germinated in Pyrex dishes with distilled water for 10–11 days. Root-tips 1 cm. long were cut on glass with a sharp razor and floated in water until used.

RESULTS

Decomposition of diethyldithiocarbamate in phosphate buffers. In a preliminary experiment it was observed that the breakdown of M/1,000 dieca was great enough to be measurable manometrically and to disturb the readings in routine measurements with barley roots. Fifty root-tips were respiring in M/15 phosphate buffer, pH 4·6, at 30° C. When the rate had been established, M/100 dieca in glass-distilled water was tipped from the side-arm to give a final concentration of M/1,000. There was an immediate reversal of the manometric curve followed by a drift back to a course parallel with the original (Fig. 1). This showed that there was a rapid decomposition of the dieca with

evolution of a gas not fixed by KOH; and that no respiratory inhibition w possible in the short time before destruction was complete.

In the absence of respiring tissues, a corresponding result was observe i.e. there was a rapid development of positive pressure for about 20 minut after which the pressure equilibrated and began to fall away. A series

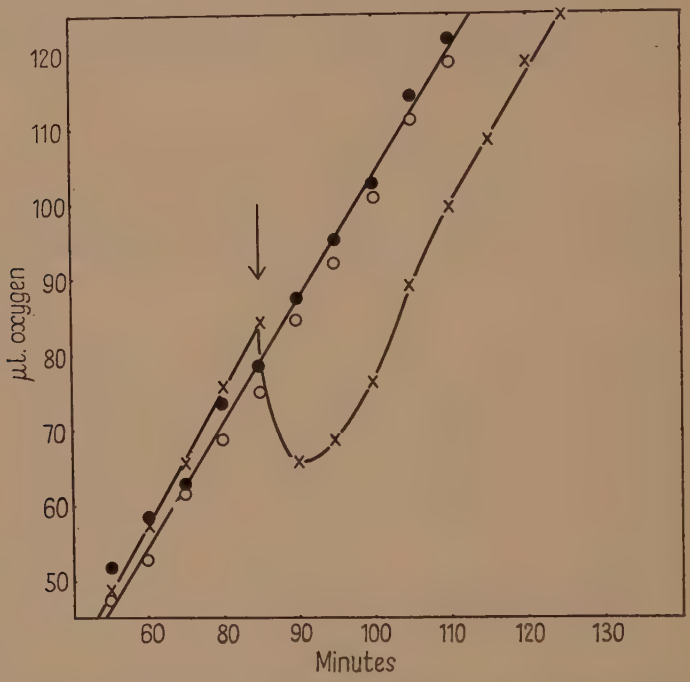

FIG. 1. Effect of 0·25 ml. of M/100 dieca on being tipped into 2·25 ml. phosphate buffer pH 4·6 surrounding 50 root-tips. The arrow indicates the moment of tipping. Crosses show the pressure changes with dieca; circles in the controls. Loss of pressure (normally O_2-uptake) is shown positive.

experiments was then performed with phosphate buffers in the main co partments of the flasks and aqueous solutions of dieca in the side-arms. Af equilibration, the dieca was tipped, and the manometric results record Experiments were run at 20·6° C. and 30° C. with buffers at pH 4·6, 6·0, a 7·0. In Fig. 2 the curves are given, corrected for the small differences in fl constants. It is evident that both acidity and temperature have a marked eff on the decomposition, and that the rate of breakdown only becomes negligi at about 20° C. and in neutral solution. This was confirmed directly by test the solutions at the end of the experiment by adding a drop of dilute cop sulphate. A good brown precipitation occurred in the neutral flask kept 20·6° C., a faint reaction at pH 6·0, and none at pH 4·6.

Products of the breakdown in acid solution are diethylamine, carb oxysulphide, and sulphuretted hydrogen (Schmidt, 1947):

$$(C_2H_5)_2\,NCSSH \rightarrow (C_2H_5)_2\,NH + COS + H_2S.$$

The presence of a secondary amine was detectable at the end of the experiments by smell and by reaction with catechol+ferricyanide or catechol+polyphenolase (Beevers and James, 1948). This remained in the solution and did not affect the pressure in the gas phase. No reaction was obtainable with lead for H_2S, which would be fixed by the KOH papers. Its removal in this

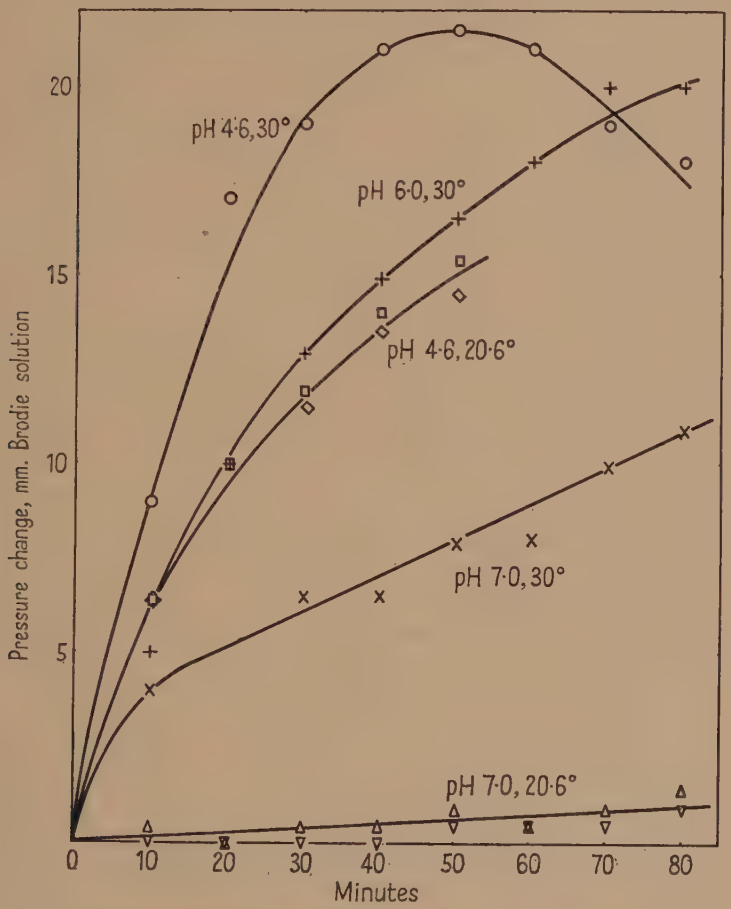

FIG. 2. Pressure changes in flasks containing phosphate buffers with 0·25 ml. M/100 dieca added at the start. Ordinates are mm. Brodie fluid (10^{-5} atm.).

way is important, since it would otherwise be expected to act as a non-specific oxidase inhibitor. The observed pressures are therefore due to the liberation of COS, which is only slowly absorbed by the KOH papers and has a water-solubility of 1 ml. per ml. at 20° C. (*Handbook of Chemistry and Physics*, 30th edition). On this basis, and assuming 0·75 of this solubility at 30° C., COS-production at 30° C. was 50·9 μl. at pH 4·6 and 51·9 μl. at pH 6·0. The quantity to be expected in 2·5 ml. M/1,000 = 56·0 μl., and the whole of the pressure appearing is therefore satisfactorily accounted for.

The action of dieca on extracted oxidases. The known metallo-oxidases—cytochrome oxidase, polyphenolase, and ascorbic oxidase—are all active a pH 7·0 and 20° C., and application of dieca may be carried out under these conditions without difficulty. Dieca has previously been reported as strongly inhibiting polyphenolase of potato (Kubowitz, 1937) and belladonna (James

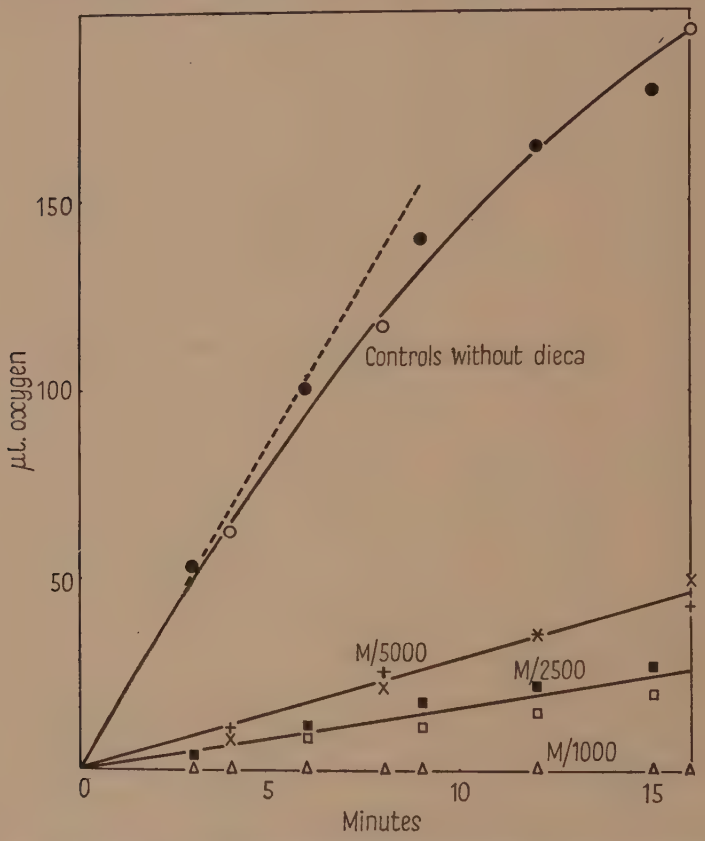

FIG. 3. Inhibition of purified mushroom polyphenolase by dieca. Percentage inhibitions calculated to the slope of the broken line.

et al., 1948) and the ascorbic oxidase of brassicas and squash (King, 1939). Mildly acid conditions and relatively high temperatures were used, and this makes the results unsuitable for quantitative estimates of the amount of inhibition caused. Kubowitz noted that the inhibition of his polyphenolase was not reversible by dialysis. Further, inhibition of the copper enzymes proves to be much faster than the rate of dieca decomposition. It is therefore possible to measure the percentage inhibition of the enzymes if the substrates are kept in the side-arms and the taps of the flasks are left open until the decomposition of the residual dieca is complete.

At 30° C. and pH 6, optimal conditions for the enzyme, inhibition of barley ascorbic oxidase was total with M/1,000 and M/2,500 dieca and $>85{\cdot}7$ per

cent. with M/5,000 dieca. At the last concentration, the rate of release of COS is so low as not to disturb the manometry appreciably.

Similar results were obtained with a highly purified polyphenolase, prepared from mushrooms by the method of Mallette *et al.* (1948), and kindly made available to us by Dr. R. M. Klein of Chicago University. The activity of this preparation was so high that it was found convenient to lower the temperato 23° C. during the measurements. In accordance with the suggestion of Adams and Nelson (1938) 5 mg. of gelatine was added to each flask containing 0·02 ml. enzyme. Five mg. ascorbic acid were added as substrate with 0·1 mg. catechol as carrier. M/1,000 dieca caused complete inhibition, M/2,500 91·4 per cent., and M/5,000 dieca 84·4 per cent. These are minimal estimates since the control rate falls away from the very first (Fig. 3).

Cytochrome oxidase is much less affected. A preliminary experiment, using a preparation made by Brown and Goddard's method (1941) and a single set of differential manometers, was run at pH 7·1 and 30° C. Spontaneous O_2 uptake without addition of cytochrome *c* was nil and inhibition after 90 minutes' shaking was 24·7 per cent. In a second experiment using the standard Warburg technique at pH 7·0 and 30° C., a preparation by Goddard's (1944) method was used. There was a just detectable oxygen consumption without addition of cytochrome *c*. Taps were closed 105 minutes after addition of dieca and there were no pressure changes in a control flask containing dieca, *p*-phenylenediamine, and enzyme but no cytochrome *c*. With the addition of 2·5 mg. cytochrome *c* per flask, inhibition due to M/1,000 dieca was 27·8 per cent. The oxygen consumption of the control in this experiment was only at the rate of 18 μl. O_2 per hour. A repeat experiment with a more active preparation showed 42·6 per cent. inhibition by M/1,000 dieca. When the concentration of dieca was reduced to M/5,000, the inhibition fell to 8·4 per cent.

TABLE I

Percentage inhibition of plant oxidases by dieca

Dieca concentration	Polyphenolase (mushroom)	Ascorbic oxidase (barley leaf)	Cytochrome oxidase (barley embryos)
M/5,000	84·4	> 85·7	8·4
M/2,500	91·4	total	26·6
M/1,000	total	total	24·7–42·6

Application of dieca to tissues

Detached barley root-tips 1 cm. long were used, since they are an absorbing zone and can be used in manometers with a minimum of cutting. Dieca tipped into phosphate buffer, pH 4·6 at 30° C., caused no inhibition of the root respiration on account of its rapid destruction (Fig. 1). Since dieca inhibition is found to be irreversible once it is caused, it is not necessary to apply the dieca under the conditions of the respiration measurements. Pretreatment under more suitable conditions is possible, after which the residual dieca can be washed away and the roots transferred to the manometers. In numerous

experiments by this method the amount of inhibition caused has remained constant during the manometric measurements (Fig. 4).

Since dieca behaves as a weak acid, it would be expected to enter the tissue more readily at mildly acid solutions than at pH 7·0. A relatively low temperature is desirable since the rate of uptake is likely to be less affected by tempera-

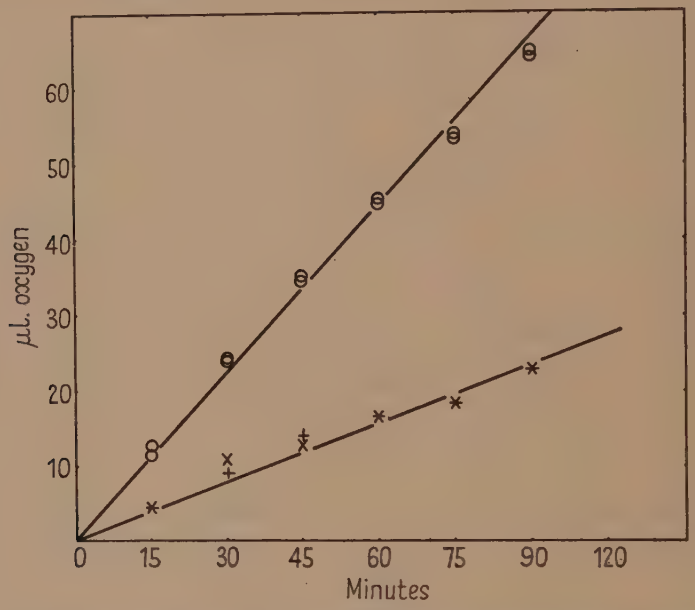

FIG. 4. Inhibition of the O_2-uptake of barley root-tips by dieca. Circles, O_2-uptake by 50 tips previously injected with phosphate buffer pH 5. Crosses, 50 tips previously injected with buffer containing M/1,000 dieca; no dieca in the manometric flasks.

ture than the rate of destruction of dieca. At room temperature, loss at pH 5·0 would still be heavy in the course of an hour (Fig. 2). To obviate this uptake may be accelerated by injection of the root-tips by first reducing the pressure on the solution containing them and then allowing it to return to atmospheric: or by frequent renewals of the solution.

A series of experiments was performed in which samples of 50 root-tips from 10 to 11-day-old barley seedlings were shaken in excess M/1,000 dieca in phosphate buffer for 1 to 2 hours. Similar samples were injected by reducing the pressure to $\frac{1}{6}$ atm. and then allowing it to return to 1 atm. These pretreatments were carried out at room temperature and different acidities. The roots were then transferred to manometric flasks and their respiration rates measured at 30° C. Injection did not increase the inhibition at either pH 7 or pH 5; lengthening the time from 1 to 2 hours was also without effect; but reducing the pH to 5 raised the inhibition from just over 50 per cent. to about 66 per cent. Reduction to pH 3·5 did not have any further effect (see Table II).

TABLE II

Inhibition by M/1,000 dieca of O_2-uptake by barley root-tips at 30° C. and pH 5·0 or 7·0

Pretreatment		Inhibition %
pH	Method	
7·0	shaken 1 hour	54·1
7·0	shaken 2 hours	50·0
7·0	injected	53·9
5·0	shaken 2 hours	69·3
5·0	injected	64·9
3·5	shaken 2 hours, changed every ½ hour	67·1

The results of the previous section (Table I) show that dieca does not differentiate satisfactorily at a concentration as high as M/1,000 but does so at M/5,000. Percentage losses of M/5,000 dieca during 2 hours' presentation to tissues at pH 5 would be very high, so frequent renewal of the solutions is necessary. Experiments were run in which barley root-tips were immersed in an excess of M/5,000 or M/2,500 dieca at pH 5. The solutions were changed 3 times at 15-minute intervals and the amount of inhibition measured manometrically in fresh phosphate buffer at pH 7·0. The inhibitions were 51·4 per cent. with M/2,500 dieca and 39·4 per cent. with M/5,000. The experiment at the last concentration was repeated with eight changes of solution lasting in all 2 hours. At the end of a 15-minute period the solution removed showed only a faintly perceptible reaction for dieca, so that the period of exposure to M/5,000 concentration was much less than the whole 2 hours. Nevertheless, the inhibition rose to 62·2 per cent., almost as high as the highest observed with 2 hours' exposure to M/1,000 dieca.

DISCUSSION

The above results indicate that dieca can be used to differentiate between extracted cytochrome oxidase on the one hand and ascorbic oxidase or polyphenol oxidase on the other, if suitable methods are used. Nevertheless, it appears doubtful whether this can be extended to all iron and copper systems. According to Albert and Gledhill, dieca is likely to react appreciably with free Fe^{++} or Fe^{+++} ions under biological conditions. The differentiation would therefore appear to depend, at least in part, upon the nature of the metal's bonding in the enzyme, and this may vary for each enzyme. The degree of purity of the enzyme may also be significant, since the inhibition recorded above for a highly purified polyphenolase is distinctly greater than was found for a crude belladonna polyphenolase (James *et al.*, 1948).

Owing to the instability of dieca in weakly acid solutions, dilute solutions must be applied to tissues with frequent renewals at room temperature. We have preferred this to the alternative method of applying the dieca to chilled tissues, because it has been shown (Barker, 1936) that prolonged disturbances of respiration rate may follow the return to normal or biologically high

temperatures from those just above zero. Application by our method has le to permanent and repeatable inhibitions of the respiration of the root-tip used. Discussion of the significance of these inhibitions in conjunction wit other essential data is reserved for a later paper; but it may be said at onc that they are inconsistent with the supposition that our barley root-tips wer dependent solely upon cytochrome oxidase for their oxygen uptake.

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An Instrument for the Continuous Determination of Leaf Thickness Changes in the Field

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SUMMARY

The paper deals with the construction and use of an instrument which measures changes in leaf thickness.

Results obtained in the field with four tree species are presented. Changes in thickness were of an individual nature for each leaf; collectively the leaves of one tree species differed in their thickness changes from those of others.

Changes in leaf thickness were mainly due to changes in the volumes of special water-storage tissues. Leaf water content changes and leaf thickness changes determined simultaneously in the laboratory were well correlated, provided that the thickness changes did not exceed those occurring in the field.

Several of the factors which influenced changes in leaf thickness were measured. The march of leaf temperature was found to be correlated most closely with the march of leaf thickness.

INTRODUCTION

THE measurement of leaf water content changes by methods commonly in use entails the destruction of the leaves. Bachmann (1922) investigated changes in leaf water content by measuring changes in leaf thickness with an assembly of levers.

The following is an account of a gear-wheel type of instrument which was used for the continuous determination of changes in leaf thickness in the field.

APPARATUS

The apparatus which was mounted on a base plate is shown in Fig. 1. The setting-screw (S), fitted with a glass platform (C), was adjustable by turning it in the holder (H). The grub-screw (G) served to secure the setting-screw in position.

The measuring mechanism of four gear-wheels (1, 2, 3, 4) was arranged between the upper (U) and the lower (L) assembly plates. On to the first gear of the train was rigidly soldered the brass arm (A), its free end carrying a glass cap (C'). The fourth wheel had a hairspring (HS) fitted to its axle and a dial (D) to its rim. A stationary indicator (J) was mounted on the lower assembly plate so that the setting of the dial could be read. A pressure of 1·2 kg./cm.^{2} was required to set the gears in motion.

All gears moved on pivots mounted either in jewel bearings or in special metal bearings as found in watches. The pivots had no play and any looseness in the mesh of the gears was counteracted by the constant pressure of the hairspring.

The magnification of movements of the brass arm was calculated from the gear ratios; one division of the dial on the fourth gear corresponded to a

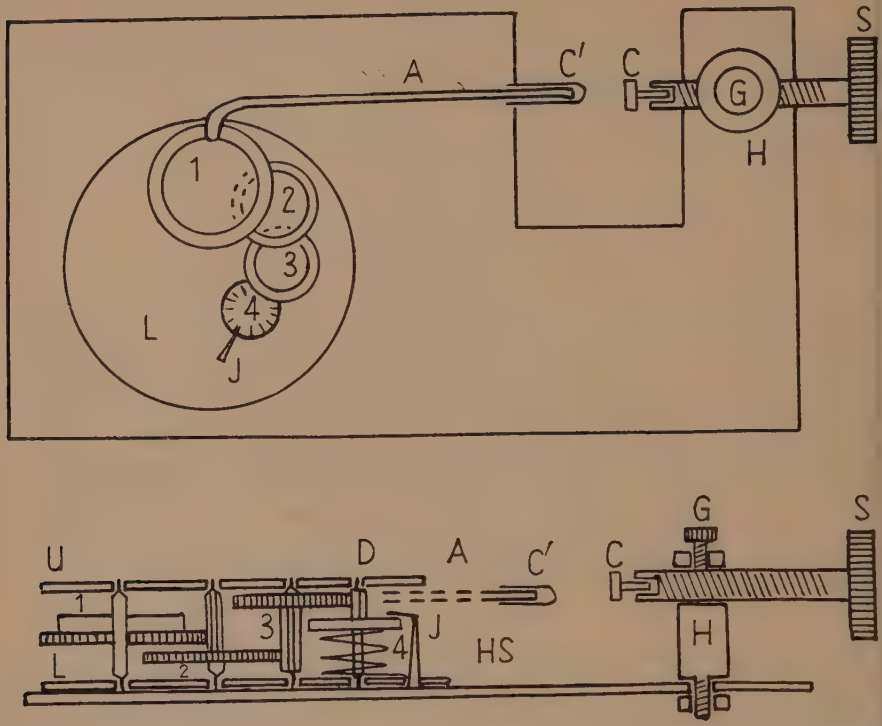

FIG. 1. Leaf thickness change meter in plan and section. A, brass arm; D, dial; H, holder; J, indicator; S, setting screw; C and C′, glass contacts; G, grub screw; HS, hairspring; L, lower assembly plate; U, upper assembly plate; 1–4, gear wheels.

movement of 0·868μ of the brass arm. 0·868 was taken as the conversion factor of dial divisions into μ. Table I shows the reproducibility of the results and the degree of sensitivity of the instrument when it was tested with feeler gauges.

TABLE I

Calibration of leaf thickness change meter with feeler gauges

Feeler gauge	Dial		Frequency	Mean		Standard deviation		Standard error	
μ	Divs.	μ		Divs.	μ	Divs.	μ	Divs.	μ
38·1	44	38·19	50	44·2	38·3	0·374	0·300	0·0483	0·0388
	45	39·06	10						
50·8	59	51·21	30	59·5	51·7	0·500	0·438	0·0647	0·0566
	60	52·08	30						
76·2	88	76·30	40	87·8	76·1	0·400	0·348	0·0565	0·0492
	87	75·50	10						

The instrument was attached to a branch or other suitable support by means of a ball-jointed clamp.

Method of use. The blade of a leaf to be investigated was placed between the glass platform (C) and the glass cap (C′). The ball joint in the attachment clamp made it possible for the instrument to be turned into any desired plane and there was no need to touch the leaf or to disturb its position.

The dial was then set, by means of the setting-screw, until it had performed one revolution.

Tests of the instrument. The results of a test carried out with a detached twig of *Cassia laevigata* are shown in Table II.

TABLE II

Leaf thickness changes of a detached twig of Cassia laevigata

Time of day	Thickness meter dial divs.	Total leaf thickness μ	Conditions
11.00	50	170	Water removed
11.25	15	139	Water supplied
12.00	52	171	Water removed
12.15	24	147	Water supplied
12.25	29	151	Twig pruned
12.30	29	151	Twig pruned again
13.00	52	171	Air current at 45° C. started
14.00	43	163	Air current continued
14.45	43	163	Water removed
15.00	25	148	Water supplied
15.20	55	174	Leaf fully turgid, undamaged

Water was alternately supplied to the twig and withheld from it. The instrument caused no detectable damage to leaves.

Dial readings were not affected by changes in temperature.

RESULTS

Investigations were carried out with four tree species which differed in their habitats.

The atmospheric moisture deficit was measured with a whirling hygrometer and expressed in millibars.

The leaf temperature was measured with a copper-constantan thermocouple which was inserted into the leaf blade.

Simultaneous measurements of leaf water content changes, by weighing, and of leaf thickness changes could only be carried out with detached leaves; therefore curves for leaf water content changes could not be included in Figs. 2–5. Results of such simultaneous measurements are stated in the text for each type of leaf.

Zizyphus mucronata Willd. Bisley, 5/4/49–7/4/49

The leaf used was exposed to the direct rays of the sun between 08.00 and 13.00 hours, remaining in the shade for the rest of the day.

The soil moisture content decreased during the 3-day period from 13·15 g. to 12·80 g. of water per 100·00 g. of dry soil at a depth of 26 cm.

Of special interest was the fluctuation of all recorded values during th second day. Leaf thickness changes were closely correlated with changes i the measured factors.

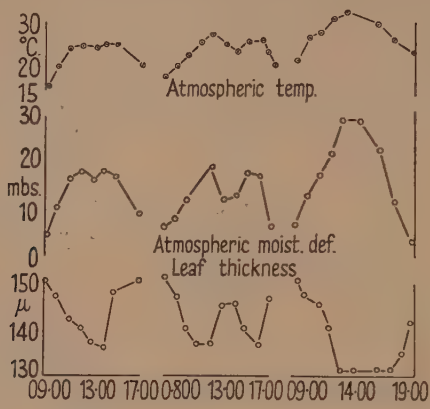

FIG. 2. Changes in atmospheric temperature and moisture deficit and changes in leaf thickness of *Zizyphus mucronata* Willd. Bisley, 5/4/49–7/4/49.

Thickness changes of up to 13 per cent. were closely correlated to lea water content changes. Every 4 per cent. change in leaf thickness corre sponded to a change of 1 per cent. in leaf water content. Correlation ceased when the leaf water content changes exceeded 3·3 per cent.

Heteromorpha involucrata Cour. Scottsville, 3/6/49–5/6/49

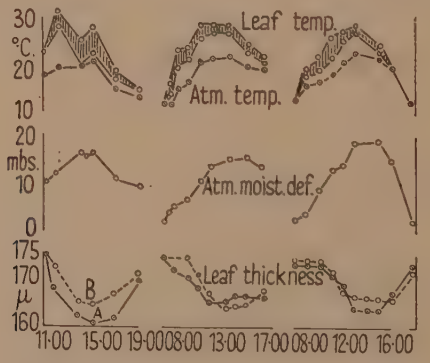

FIG. 3. Changes in atmospheric temperature and moisture deficit and changes in temperature and thickness of leaves of *Heteromorpha involucrata* Cour. Scottsville, 3/6/49–5/6/49.

Leaf *A* was exposed to direct insolation between 10.00 and 14.00 hours leaf *B* was intermittently in direct sunlight and shade cast by other leaves.

Changes in thickness of up to 9 per cent. were closely correlated with lea

water content changes. Every 1 per cent. change in leaf water content corresponded to a change of 4·5 per cent. in leaf thickness. Correlation ceased when the leaf water content changes exceeded 1·8 per cent.

Gymnospora buxifolia Szysyzl. Bisley, 21/6/49–23/6/49

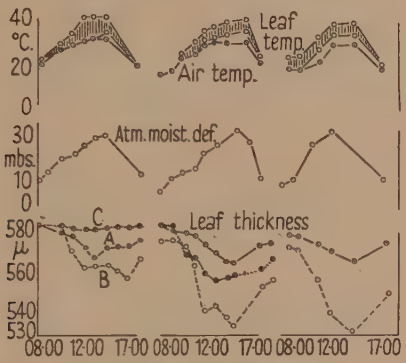

FIG. 4. Changes in atmospheric moisture deficit and temperature and changes in thickness and temperature of leaves of *Gymnospora buxifolia* Szysyzl. Bisley, 21/6/49–23/6/49.

Leaf *A* was exposed to the direct rays of the sun between 08.00 and 14.00 hours; leaf *B* between 10.00 and 14.00 hours, leaf *C* was in shade on the first day but on the second day it was exposed to the sun between 09.00 and 13.00 hours.

The maximum leaf temperature of 40·0° C. was 11·0° C. above the temperature of the atmosphere. The temperature of leaf *B* only was measured.

While maximum values for leaf and air temperatures were measured on the first day, the greatest changes in thickness were recorded on the third day.

Changes in thickness of up to 9 per cent. were closely correlated with changes in leaf water content. Every 1 per cent. change in leaf water content corresponded to a change of 7·5 per cent. in leaf thickness. Correlation ceased when the change in leaf water content exceeded 1·2 per cent.

Xymalos monospora Baill. Boughton, 30/7/49–1/8/49

Both leaves investigated were shade leaves, being exposed to the direct rays of the sun for a few minutes at a time several times during the day. Leaf *A* was exposed to direct sunlight less often and for shorter intervals than leaf *B*.

The fluctuations in the values of the atmospheric moisture deficit during the first day were due to warm air currents passing.

Fluctuations of 12·0° C. in the leaf temperature were recorded within short intervals of time; the leaf temperature rose in a very short time owing to the direct insolation of the leaves.

Close correlation of leaf temperature changes with leaf thickness changes was shown at 14.00 hours on the first day, when both these values remained constant, while the atmospheric moisture deficit and the atmospheric temperature had increased. Decreases in leaf thickness were preceded by increases in leaf temperature.

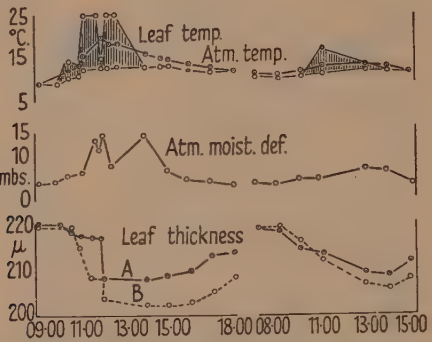

FIG. 5. Changes in atmospheric temperature and moisture deficit and changes in temperature and thickness of leaves of *Xymalos monospora* Baill. Boughton, 30/7/49–1/8/49.

Changes in thickness of up to 8 per cent. were closely correlated with changes in leaf water content. Every 1 per cent. change in leaf water content corresponded to a change of 7 per cent. in leaf thickness. Correlation ceased when the change in leaf water content exceeded 1·0 per cent.

DISCUSSION

With detached leaves, simultaneous measurements of leaf water content and leaf thickness showed that these were closely correlated; provided that for each type of leaf, changes in thickness did not exceed in magnitude those measured in the field. Maximum changes in leaf thickness recorded in the field varied between 8 and 13 per cent. of the total thickness, according to the type of leaf investigated; maximum changes in leaf water content in the field varied between 1 and 3·3 per cent. of the total leaf water content. These figures agree with the results obtained from simultaneous measurements of leaf water content changes and leaf thickness changes with detached leaves.

On account of the 'absorption-lag' as postulated by Kramer (1938) and also on account of the resistance of leaf tissue to water movements as shown by Mer (1940), changes in leaf water content occur during periods of transpiration, even though water is available to the plant. Thus changes in soil moisture content, as measured in the field, were found to have no influence on changes in leaf water content; this agrees with Portsmouth's (1937) findings. Thickness changes were also recorded with leaves of a tree growing next to a stream of water.

The orientation of leaves to the sun and their inclination to the sun profoundly influenced the march of leaf temperature. Changes in leaf thickness were correlated closely with changes in leaf temperature, which confirms Nutman's (1941) views.

The epidermal water storage tissue of *Zizyphus mucronata* leaves measured up to 72μ out of a total of 152μ. The comparatively large thickness changes (13 per cent.) of these leaves were probably due to the partial collapse of the epidermal cells (Haberlandt, 1914). Although the percentage change in thickness of leaves of *Xymalos monospora* was the smallest of the leaves investigated, it represented the most immediate reaction of leaves to changing external conditions, such as occurred in the forest when the leaves were exposed to direct sunlight for a few minutes at a time.

The percentage changes in thickness of all four leaves, when allowed to wilt in the laboratory, were consonant with changes in thickness measured in the field. The use of the instrument described contributed to the methods of studying water relations of plants in the field, especially to the study of changes in leaf water content during short intervals of time.

ACKNOWLEDGEMENTS

Throughout the investigation encouragement and help was given by Professor A. W. Bayer. In connexion with the anatomical studies the late Dr. B. S. Fisher gave valuable advice. This assistance is hereby gratefully acknowledged.

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Preparation of Radioactive Starch, Glucose and Fructose from $C^{14}O_2$

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SUMMARY

Radioactive starch, glucose and fructose have been prepared from tobacco leaves after assimilation of $C^{14}O_2$. The apparatus used for photosynthesis consisted of a shallow Perspex leaf chamber connected to a closed gas system, in which $C^{14}O_2$ was generated from $BaC^{14}O_3$. Six leaves, area 14 to 18 sq. dm. when exposed to bright sunlight with an initial CO_2 concentration of 8 to 10 per cent., assimilated 3·35 g. of $C^{14}O_2$ in 8 to 10 hours. At least 80 per cent. of the $C^{14}O_2$ supplied appeared in the leaves as starch and sugar and over 80 per cent. of the radioactivity was accounted for in these carbohydrates. The specific activity per m. atom of carbon of the isolated products was 85 to 90 per cent. of that of the $C^{14}O_2$. Small amounts of radioactive carbon were also incorporated in the leaf protein and in the cellulose, hemicellulose and polyuronides.

PUTNAM, Hassid, Krotkov and Barker (1948) have shown that radioactive starch and sugars can be isolated from tobacco leaves after 24 hours' exposure of detached leaves to light in the presence of $C^{14}O_2$. The photosynthesis apparatus described was designed to accommodate a single leaf, which was supplied with approximately 0·25 μc. of C^{14} as CO_2 by the action of lactic acid on 500 mg. of $BaC^{14}O_3$. This procedure has now been adapted to the preparation of larger amounts of radioactive carbohydrates by constructing an apparatus to accommodate 6 to 12 tobacco leaves. Six leaves were found to elaborate at least 2 g. of starch and sugar when exposed to bright sunlight for 8 to 10 hours in the presence of $C^{14}O_2$.

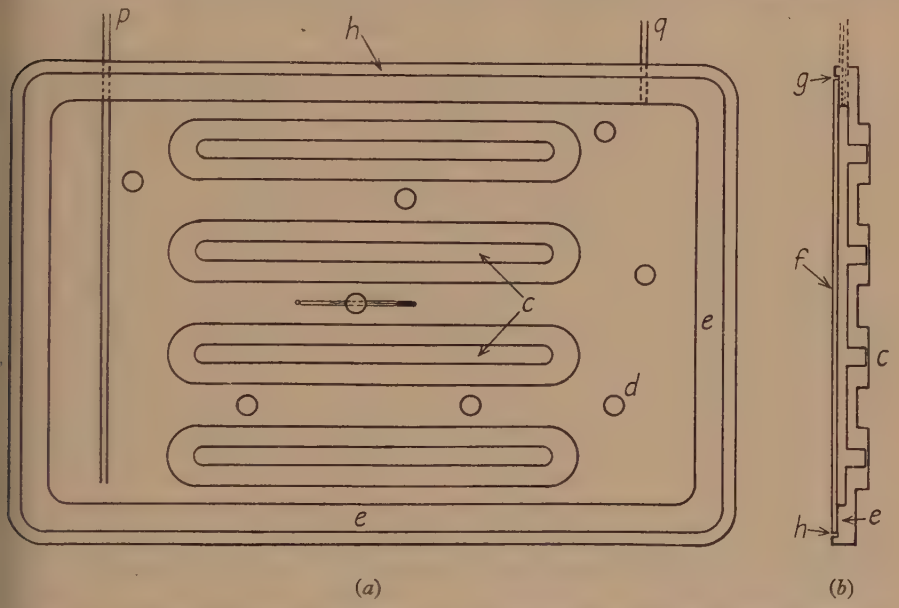
MATERIAL

Tobacco ('White Burley') was grown at first in 6-in. pots in soil in a warm greenhouse. Six to eight weeks after sowing symptoms of nitrogen deficiency appeared and these were corrected by transplanting into 10-in. pots and giving two doses of 0·25 g. $NaNO_3$ and 0·1 g. K_2HPO_4 per pot with an interval of a week between the doses. In the nitrogen-deficient plants periods of 5 to 7 days in the dark failed to remove all the starch save in the youngest leaves. This slow rate of starch loss in the dark appears to be associated both with nitrogen deficiency and with leaf age. In plants grown with adequate nitrogen supply in 10-in. pots the two most recently emerged leaves at any time were destarched after 48 hours in the dark, but lower leaves required longer dark periods. For the photosynthesis experiments, therefore, the plants were grown

with sufficient nitrogen throughout and only the two most recently emerged leaves used. The plants were kept in the dark for 60 hours, 3 nights and 2 days, prior to detaching the required leaves. Absence of starch was confirmed by removing two small disks from each leaf with a cork borer and testing with iodine. Leaves still containing any starch were rejected. The selected leaves were detached from the plants, weighed, and introduced into the leaf chamber.

APPARATUS FOR PHOTOSYNTHESIS

Diagrams of the leaf chamber and of the gas circuit employed are presented in Figs. 1 and 2. The leaf chamber, which was made of Perspex, is shown in plan in Fig. 1(*a*) and in section in Fig. 1(*b*). The dimensions of the base were



(*a*) (*b*)

FIG. 1. (*a*) Plan of Perspex leaf chamber. It is connected to the gas circuit at *p* and *q*. (*b*) Transverse section of leaf chamber. See text for description.

50 by 80 cm.; in it were sunk four troughs (*c*) 2 cm. deep and 2·5 cm. wide. These troughs were filled with distilled water into which the leaf petioles dipped. Strips of cellophane tape ensured that the petioles remained below the surface of the water. A number of Perspex blocks (*d*) was placed about the base of the chamber to support the lid and into one of these a thermometer was inserted, so that temperatures inside the chamber could be recorded. Surrounding the base was a flange (*e*) 4 cm. wide upon which the lid (*f*), a Perspex sheet 0·8 cm. thick, rested. When sealed, the depth of the chamber from lid to the main base was 1·5 cm. When the lid was in position there was a groove (*g*) 0·6 cm. wide between it and the outer edge (*h*) of the chamber. Inlet and outlet tubes for the gas stream (*p* and *q*) were arranged so that the gas entered and left the leaf chamber by diagonally opposite corners.

The gas circuit (Fig. 2) consisted of a litre Pyrex flask (*a*), in which $C^{14}O_2$ was generated, connected to a burette (*b*), to a small diaphragm pump (*c*), and to the leaf chamber by a gas line carrying a manometer (*e*), which recorded the pressure in the circuit. Gas could be circulated through the flask, then via the pump through a wash bottle (*f*) and by a suitable arrangement of taps through two further wash bottles (*g* and *h*), which contained

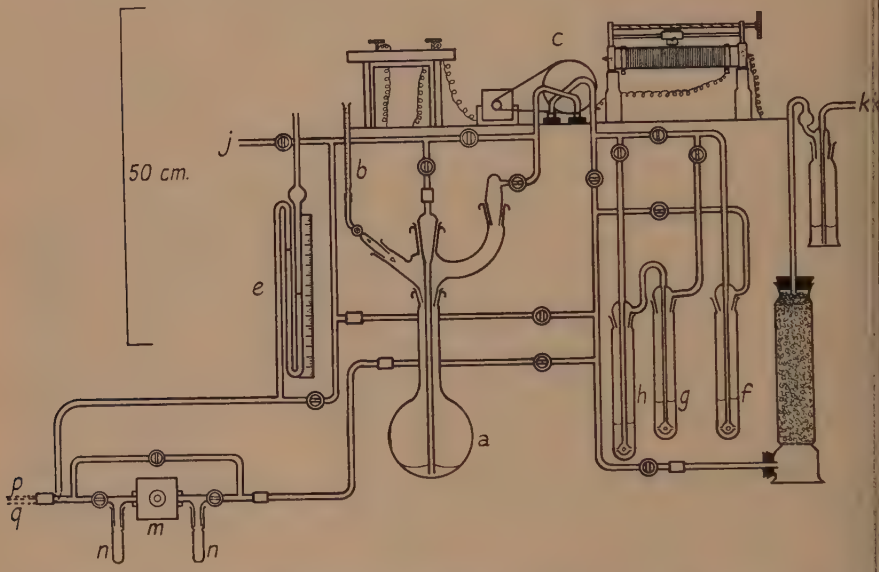


FIG. 2. Gas circuit for generating and circulating $C^{14}O_2$. It is connected to the leaf chamber by *p* and *q*. See text for description.

either indicator (brom-cresol purple) to check the CO_2 concentration, c NaOH when it was necessary to absorb excess CO_2, and finally through th leaf chamber and back into the flask. The system could be connected to vacuum source at *j* and to a source of CO_2 free air at *k*. One lead to the lea chamber was fitted with a by-pass into which was introduced a sma brass gas chamber (*m*) attached to drying-tubes (*n*). This chamber, which ha a volume of about 20 ml., had a mica window and by placing an end-windo Geiger counter over it, comparative counts of the radioactivity of samples c the circulating gas passed into the chamber could be made.

At the outset of an experiment 50 ml. of water were placed in the flas and then 15 g. of $BaC^{14}O_3$ introduced enclosed in pieces of glass tubing eac containing about 5 g. of carbonate. One end of each piece of tubing wa sealed with gummed paper to facilitate weighing out the material and whe the tubes were submerged in water the papers became detached, allowin free circulation of gas and liquid over the carbonate. The flask was the placed in position. The flange of the leaf chamber and edges of the lid we then well greased with resin cerate and six leaves placed in position in th

roughs. The lid was now placed in position and kept firmly in place by welve small G-clamps screwed down around the edge of it. The leaf hamber was sealed by pouring hot resin cerate into the groove (Fig. 1(*b*), *g*) urrounding the lid and allowing the wax to set. The efficacy of the seal was ested by reducing pressure within the circuit by withdrawing gas at *j* and bserving the manometer level. If required the apparatus could now be swept ut with CO_2 free air entering through absorbing towers at *k*. Since atmoheric CO_2 initially present in the 7-l. volume of the system represented nly 0·1 per cent. of the CO_2 subsequently generated during these experients, this step was usually omitted. The internal pressure was now adjusted o that when two-thirds of the total amount of CO_2 to be supplied had been eleased the pressure would rise to a value slightly below atmospheric level. n the event of a leak developing, air would thus at first enter the circuit ather than $C^{14}O_2$ leak out. The pump was then switched on and at once 6 ml. f 80 per cent. lactic acid run in through the burette (*b*) followed by a further ml. during the next hour. When 15 g. of $BaCO_3$ was used this was sufficient o decompose two-thirds of it and to produce an initial concentration of CO_2 f 8 to 10 per cent. Gas was drawn down through the tube leading to the ottom of the flask and carried the CO_2 being evolved towards the lead to the ump. The rapid evolution of gas formed a fine spray which carried some id into the circuit; consequently the exit from the flask was plugged with lass wool to trap as much as possible of the spray, and the lead from the ump was connected to the wash bottle (*f*), containing water which removed ny remaining acid. The gas now passed into the leaf chamber by the gas line *p* and finally back into the flask by the line (*q*). The flask and the chamber ould both be by-passed without breaking the closed circuit.

The gas was circulated until the CO_2 level had fallen to about 0·01 per cent., judged by passing samples through the bottles (*g* and *h*) containing indiator, at intervals of about half an hour. The course of CO_2 absorption was lso followed by introducing samples of gas at intervals into the chamber (*m*) nd after drying, recording comparative estimates of radioactivity. The inernal pressure was then again reduced by removing gas at *j* and absorbing in NaOH to trap traces of $C^{14}O_2$. A large excess of lactic acid was then run to the flask to ensure decomposition of the remaining carbonate and circulaon of gas continued until once more only traces of $C^{14}O_2$ remained. The ternal pressure was adjusted if too low when no further CO_2 was being eased, by introducing CO_2 free air at *k*. Before opening the leaf chamber. ash bottles containing NaOH were fitted at *g* and *h* in place of those conning indicator and any residual $C^{14}O_2$ removed by passage of the gas rough these wash bottles. The absorbed $C^{14}O_2$ was recovered as $BaC^{14}O_3$ nd weighed.

The whole apparatus was set up in a greenhouse, and operated on sunny ys in June and July from about 7 a.m. until all the $C^{14}O_2$ had been assimied, a period of 8 to 10 hours with the leaf areas and CO_2 concentration osen. Temperatures in the chamber ranged from 14° C. to 18° C. at 7 a.m.,

and rose to 30° C. to 35° C. at noon, and then fell again to about 25° C. by 4 t 5 p.m. Under these conditions six leaves with a fresh weight of 30 to 60 g assimilated at least 3·4 g. of CO_2. No attempt was made to find out how nea maximal this amount was.

ISOLATION OF STARCH

At the end of the assimilation period the leaves were removed from th chamber and at once transferred to an Atomix blender containing 150 ml. o 0·02 M. phosphate buffer at pH 7·0 and 25 ml. of toluene. A wire frame acros which a piece of muslin was stretched was fitted inside the top of the blende jar and the leaves disintegrated for 3 to 5 minutes. The presence of the toluen leads to the formation of an emulsion, which was poured off through th muslin into a 200-ml. centrifuge bottle, the leaf residue being retained in th jar. This process was repeated five times by successive additions of water o buffer through the muslin, but omitting the toluene. After six extractions th leaf residues contained only traces of starch. The extracts were then centri fuged for 30 minutes at about 2,700 r.p.m. The first extract containing mos of the starch and sugars separated into three layers. Starch was at the botton a brown slightly opaque liquid layer above and at the top a semi-solid mas of lipids, green pigments and toluene. When the bottle was gently tilted, th lipid mass rested on the side and the liquid layer could be poured off. Th lipids, &c., were then removed with a plastic spoon previously bent to fit th neck of the bottle. Some of the later extracts required removal of small amounts of material from the top in this way, depending upon the efficienc of the first filtration of the emulsion through the muslin. Leaf age als affected the amount of this fraction and in late emerged leaves the wax co tent was so low that the top layer was virtually a solution of pigments in tol ene and had to be removed with a syringe pipette. The lipid material was s aside; if desired, a little occluded starch could be recovered from it by di persing in about a litre of water and centrifuging.

The starch precipitates were collected into one bottle and washed on or twice with water by centrifugation. The starch was then suspended about 150 ml. of water and allowed to sediment. The grains of tobacco le starch are very small and sediment slowly, so that cell fragments remaini settle first and the starch suspension can be removed from the top. By repea ing the sedimentation process starch was freed from contaminating fragmen it was then centrifuged, washed with alcohol and ether, and dried *in vacu*

ISOLATION OF FRUCTOSE AND GLUCOSE

The aqueous extract usually contained only fructose and glucose, sucro being inverted during the disintegration process. The brown extract w boiled to coagulate protein, filtered, and the filtrate evaporated under reduc pressure, not above 40° C., to a volume of about 10 ml. An equal volume absolute alcohol was added dropwise with stirring and the resulting preci tate, mostly salts, removed by centrifugation. The precipitate was wash

once or twice with 50 per cent. alcohol and the washings and the supernatant liquid evaporated, as before, to a gum. In these experiments the reducing sugar content of this gum was of the order of 600 mg. The gum was taken up with 4 ml. of water, giving a total volume of 5·5 ml. and the solution transferred with a syringe pipette to a column 21 × 3·5 cm., holding 65·6 g. of a 50/50 B.D.H. activated charcoal/celite mixture (Whistler and Durso, 1950), having a bed volume of 140 ml. The flask in which the sugar solution had been evaporated was washed with four successive lots of 1 ml. of water and these washings were also transferred to the column.

The column was now eluted with water, the first bed volume together with the next 50 ml. of eluate, which contained most of the remaining salts but no sugar, were discarded. The sugars were eluted in the ensuing 300 ml. and the course of elution followed by observing the copper-reducing power of 1 ml. or less of successive 25-ml. fractions and also by drying two drops from each fraction and making comparative counts of radioactivity. In these experiments more than 50 per cent. of the sugar was found in one 25-ml. fraction between the 65th and 90th ml. of the 300 ml. required for complete elution. After 300 ml., no reducing power could be detected in 1 ml. of eluate, but as a rule the amount of radioactivity remained somewhat above the background value. The eluate was water-clear and when tested by paper chromatography, using butanol/acetic acid as the developing solvent (Partridge, 1948) and spraying with benzidine/trichloracetic acid mixture (Bacon and Edelman, 1951), it showed only glucose and fructose spots. Furthermore, only traces of radioactivity were found on areas of the paper other than the sugar spots.

The eluate containing the sugars was evaporated as before to small volume and then dried *in vacuo* to a gum. It was then taken up in 5 ml. of water and any contaminating charcoal/celite mixture removed by centrifugation. The aqueous liquid was again evaporated to dryness and the gum taken up in a little water and made to 76 per cent. alcohol in a total volume of 10 ml. The alcohol solution was transferred as before to a column 30 × 2·7 cm. containing a 5/1 Florex XXX/Celite mixture (Wolfrom and Shilling, 1951) for the separation of glucose from fructose, by elution with alcohol.

The Florex XXX was used as supplied and, as noted by Mowery (1951), used in this way most of the glucose can be eluted by 95 per cent. alcohol, but removal is never quite complete. Fructose is eluted more slowly, but after the glucose is gone the rate of fructose elution can be conveniently hastened by further elution with 80 per cent. alcohol. There is, however, a long 'tail' of glucose which contaminates the fructose fraction. A further difficulty encountered was the manifestation of Florex particles in the effluents when these were evaporated, which proved rather tiresome to remove. It would appear to be desirable to adopt Mowery's (1951 *a*) precaution of screening the Florex to remove the fine particles before packing the column, by which procedure also improved separation of the sugars is claimed.

In operating the column the flow rate was increased by applying a positive pressure of about 1 atmosphere to an eluant reservoir attached to the column

(cf. Mowery, 1951) and attaching the receiving flask to an evacuated desic cator giving a negative pressure of about 68 cm. of mercury. After the passag of about 385 ml. of 95 per cent. alcohol, glucose appeared in the eluate. Suc cessive 60-ml. fractions were now tested on paper with the benzidine spray With the amounts of glucose separated here, there was a maximum amount i the fraction between 480 and 530 ml. and glucose was only just detectable i that from 840 to 900 ml. After the passage of 900 ml. of 95 per cent. alcoho the eluant was changed to 80 per cent. alcohol and 1,500 ml. passed throug the column to ensure fructose removal. As a rule only traces of fructose wer detected after about 900 ml. of 80 per cent. alcohol had run through th column.

The alcohol fractions containing the sugars were evaporated as before an taken up in water when the Florex particles dispersed. By adjusting t 70 per cent. alcohol in a total volume of 15 ml. most of the Florex coagulate and was removed by centrifugation. The supernatant liquids were evaporate to dryness *in vacuo* over P_2O_5. For crystallization, the glucose was transferre in 0·5 ml. of water to a graduated centrifuge tube and absolute alcohol adde to 92 per cent. After a week the mother liquor was removed from the crystal which were washed with 1 ml. of absolute alcohol and dried *in vacuo*. Th mother liquor and washings were evaporated again to dryness and two furth crops of crystals obtained by solution in smaller volumes of 92 per cen alcohol. The fructose, which was slightly brown, was dissolved in 0·5 m of water and treated twice with 2 ml. of toluene which removed most the colour. The solution was then transferred to a charcoal/celite column, 60 1·5 mm., which had been washed with water. The liquid was drawn throug the column by gentle suction and successive 0·2 ml. washings of the dis were also passed through the column. In this way 2·5 ml. of colourless elua was obtained. This was dried over P_2O_5 and weighed as a gum.

Yields of sugar for one complete experiment are presented in Table I. will be seen that there is a loss of 13 per cent. of the reducing power on passa through charcoal, 76 per cent. of the initial reducing power was recover from the Florex column and the combined yields of crystalline glucose an purified dry fructose represented 68 per cent. of the estimated reducing sug in the crude aqueous extract.

TABLE I. *Isolation of glucose and fructose from aqueous extracts of Tobacc leaves. (Expt. of 17/7/51; see also Table II)*

	Estimated reducing sugar (mg.) in			
	aqueous extract	eluate from charcoal	eluates from Florex	mg. suga isolated
Reducing sugar	550	478	420	379
Glucose	—	275	240	229
Fructose	—	203	180	150*
% recovery of reducing sugar	—	87	76	68

* Separation of the glucose contaminant (see p. 331) was finally effected by pa chromatography (Bacon and Edelman, 1951).

RESULTS OF ASSIMILATION EXPERIMENTS

Data for three experiments carried out in July 1951 are presented in Table II, *a* and *b*. About 15 g. of $BaC^{14}O_3$ were used in each case, equivalent to 3·35 g. of CO_2 or 2·28 g. of hexose. At the end of each experiment the small amount of CO_2 remaining unassimilated was recovered as $BaCO_3$ and allowance has been made for the amounts thus recovered in calculating the figures of Table II. The leaves used were obtained from a batch of plants sown 13 weeks before the time of the first experiment recorded here. The smaller leaf area of later emerged leaves accounts for the sequence of fresh weights and leaf areas in Table II*a*. The smallest area used, 14 sq. dm., was adequate to assimilate the 3·35 g. of CO_2 supplied, under these particular environmental conditions. The initial assimilation rates when CO_2 was released into the system and concentration rose to about 10 per cent. must have been much higher than the averages recorded in Table II*a*, because the necessity of removing as much CO_2 as possible by assimilation resulted in a rather long period of CO_2 uptake from very low concentrations. At least 80 per cent. of the CO_2 supplied was converted to carbohydrate (see Table II*b*; cf. Smith, 1944) and the ratio of starch to sugar was high when leaf area was low. Estimated sugar concentrations in small leaf disks removed before the leaves were disintegrated indicated that sugar concentrations rose to about the same value of about 1·8 per cent. of the fresh weight in all three experiments, the balance of carbohydrate formed appearing as starch.

TABLE II

Assimilation of $C^{14}O_2$ by detached tobacco leaves exposed to bright sunlight for 8 to 10 hours. Temperature inside leaf chamber 14° to 18° at 7 a.m. rising to 30° C. to 35° C. at noon

(*a*)

	Date	Fresh weight per leaf (g.)	Area of 6 leaves (sq.dm.)	Mean assimilation rate (mg. CO_2 sq.dm./hr.)	CO_2 assimilated (g.)	Starch isolated (g. hexose)	Sugar estimated in aq. extract (g. hexose)	Starch + sugar (g. hexose)
1.	19/6/51	9·5	20·0	14	2·28	1·03	0·70	1·73
2.	3/7/51	8·3	18·0	19	2·30	1·25	0·60	1·85
3.	17/7/51	6·7	14·4	23	2·26	1·70	0·55	2·25

(*b*)

	Percentages of carbon dioxide supplied recovered as		
	Starch	Sugar	Total*
1.	45	31	76
2.	54	26	80
3.	5	24	99

* These values are based on the starch isolated and the estimates of sugar in the aqueous leaf extract and are therefore minimal yields.

TABLE III

Radioactivity of starch and sugars isolated from tobacco leaves

	μc. supplied in $C^{14}O_2$	Per cent. in isolated starch or sugar	Specific activity μc. per m. atom C. $C^{14}O_2$	starch or sugar	Ratio of sp. activities starch or sugar/CO_2
			a. Starch		
19/6/51	91·9	45	1·21	1·03	0·85
3/7/51	92·9	48	1·21	1·10	0·90
17/7/51*	14,000	64	183·00	157·00	0·87
			b. Sugar		
17/7/51*	14,000	18·5	183·00		
			glucose	141·00	0·80
			fructose	158·00	0·86

* For experiment of 17/7/51 recovery of activity in starch and sugar 82·5 per cent.

Measurements of radioactivity for the three experiments are recorded in Table III, *a* and *b*. The specific activity per m. atom of carbon of the isolated starch and sugars was 85 to 90 per cent. of that of the CO_2 supplied to the leaves. The proportion of the activity recovered in starch varied from 45 to 64 per cent., according to the relative amount of starch produced. For the experiment of 17/7/51 the specific activity of both fructose and glucose was not significantly different from that of the starch. On the basis of the activities of the isolated sugars and on the amount of sugar estimated to be present in the crude aqueous extract, 18·5 per cent. of the total radioactive carbon had become incorporated in sugar, making a total recovery of 82·5 per cent. as starch and sugar. The activity of the sugar actually isolated was 12·6 per cent. of the total.

The residual leaf material which contained traces of starch was digested with α-amylase and from the starch-free residue preparations of cellulose, hemicellulose and polyuronide were made. These preparations were all radioactive and contained approximately 2 per cent. of the total activity. The protein precipitate obtained from the aqueous extract was washed again with water and then exhaustively extracted first with ether and then with 95 per cent. alcohol. The protein was then hydrolysed with 6N. HCl and the hydrolysate subjected to two-dimensional chromatography on paper, using phenol and collidine as the developing solvents. After spraying with ninhydrin the paper was cut up into 2-cm. squares and each square tested for radioactivity. Activity was confined to the positions in which amino acids occurred as defined by the ninhydrin colour, except for a small amount near the solvent fronts. These observations are taken to be a qualitative demonstration of incorporation of a small proportion of radioactive carbon from carbon dioxide assimilated by detached leaves into protein and into cellulose type carbohydrates. Whether this is brought about by overall synthesis or by exchange cannot be determined from the present data because some of the leaves

used were not quite fully expanded and so the possibility of a little growth is not excluded.

We are indebted to Dr. G. Popjàk of the National Institute of Medical Research for his stimulating help in initiating this work and for the gift of the gas sampling chamber incorporated in the assimilation apparatus. We also wish to thank Mr. F. J. Richards of this Institute for chromatographing the protein hydrolysate, Dr. M. Tracey of Rothamsted Experimental Station for preparing the cellulose samples, Mr. F. Smith who constructed the leaf chamber and, finally, Professor F. G. Gregory, F.R.S., for his constant encouragement and advice.

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Organic Acid Metabolism of *Sedum praealtum*

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SUMMARY

1. The organic acids present are citric, *iso*citric, and *l*-malic, with a small residue of unidentified acids.

2. The diurnal variation in acidity is due chiefly to changes in malic acid, with a parallel fluctuation shown by citric acid. Under these conditions *iso*citric acid shows little change.

3. The importance of carbon dioxide during acidification is confirmed, and it is shown that at room temperatures or higher the CO_2 produced in respiration is sufficient to produce maximum acidification. At lower temperatures the supply of CO_2 limits acid production.

4. In the absence of oxygen no acidification occurs, but even small quantities (approx. 1 per cent.) are sufficient to cause some acid production.

5. Complete balance-sheets are presented for acids, carbohydrates, CO_2 and oxygen for leaves maintained in the dark at high and low temperatures. As acids are produced there is a corresponding loss of carbohydrate (chiefly starch). A scheme of reactions is suggested to explain the experimental results.

INTRODUCTION

THE problem of acid metabolism in the Crassulaceae has excited interest for many years, and reviews of the literature have been given by Bennet-Clark (1933, 1949), Thomas (1949), Vickery and Pucher (1940).

By the introduction of specific methods for the estimation of the various organic acids, Pucher, Vickery, *et al.* have demonstrated that the acids present in *Bryophyllum* are malic, citric, and *iso*citric, with small traces of oxalic acid. The malic acid occurs as the *l*-isomer (Pucher, 1942) and the so-called 'Crassulacean malic acid' has been identified with *iso*citric acid (Pucher and Vickery, 1942).

The source from which organic acids are formed has also excited interest and has been shown to be carbohydrate in nature, starch being the chief compound concerned (Pucher *et al.*, 1947; Wolf, 1938).

Difficulties are encountered in attempting to draw up balance-sheets for the conversion of carbohydrates to acids during the diurnal fluctuation owing to the formation of fresh carbohydrate during the light period. Many of the results to be described were, therefore, obtained from leaves maintained in complete darkness, in order to obviate this factor. In the absence of light carbohydrates are converted to acids, and this has, therefore, been the stage most fully investigated.

Earlier experiments (Wolf, 1938; Pucher *et al.*, 1948) have shown that the amounts of acid formed in the dark are dependent upon temperature. At a high temperature less acid is formed than at a low temperature.

If leaves are transferred, while still in the dark, from a low to a high temperature, organic acids disappear, and vice versa on transference from a high to a low temperature. Use has been made of this fact in the present investigation in order to bring about the acid disappearance phase in the dark (i.e. in the absence of photosynthesis).

Another factor in acid production is the presence or absence of carbon dioxide. The evidence for the importance of this gas has been very fully investigated and reviewed by Thomas (1949), and experiments with C^{14} have proved that CO_2 is fixed in malic acid (Varner and Burrell, 1950; Stutz and Burris, 1951). Experiments of Bonner and Bonner (1948), Thurlow and Bonner (1948), and of Thomas and Beevers (1949) show that in members of the Crassulaceae acid production is directly dependent on the partial pressure of carbon dioxide in the surrounding air. It appears that one mole of carbon dioxide is fixed in one mole of malic acid.

Carbon dioxide changes have therefore been investigated at the same time as those of acids and carbohydrates, and full balance-sheets for these components under a variety of conditions will be presented in this paper. This work was started before the very complete chemical analyses on *Bryophyllum* by Pucher, Vickery, *et al.* had been published; it therefore provides independent evidence that *Sedum praealtum* exhibits a remarkable similarity in behaviour.

MATERIAL AND METHODS

Plants of *Sedum praealtum* were grown outdoors during the summer months, and were maintained during the winter in a temperate greenhouse.

For any one experiment nearly fully-expanded leaves were cut, and placed in shallow dishes with their petioles beneath moist cotton-wool for the duration of the experiment. This treatment ensured equal illumination for all leaves and prevented excessive water loss.

The samples used for the determinations were always large (20 to 25 g.), and in any one experiment leaves from the same plant were used; or if this proved impracticable, thorough mixing of equal quantities of leaves from a number of plants was substituted. In view of the lengthy determinations involved, it was found impossible to deal with more than a single sample at any one time. A few preliminary experiments, however, indicated that the variation between small samples (5 g.) was not greater than 10 per cent., and that between larger samples (20 to 25 g.) was not greater than 6 per cent.

Leaves were crushed in a mortar and ground to a fine consistency. Water was added and the pulp boiled for 5 minutes. The solution was cooled, filtered, and made to volume (usually 100 ml.). Details as to the completeness of extraction by this technique are given by Bennet-Clark (1933).

This solution was used for the determinations of malic acid, citric acid, *iso*citric

acid, titratable acidity, total acids, fermentable and non-fermentable reducing sugars and sucrose, using the following methods:

Titratable acidity. A suitable aliquot of the solution was titrated to phenolphthalein end-point with standard caustic soda solution. Results were expressed as mg. eq./100 g. fresh weight of leaves.

Total acids. The method used employed precipitation of the acids with basic lead acetate solution and is based on that described by Bennet-Clark (1933). The acids are precipitated as the lead salts which are then removed by centrifuging, and the free acids liberated by passing hydrogen sulphide. Excess gas is boiled off, and the acids titrated against standard alkali, as in the determination of titratable acidity. This method determines both organic acids and any inorganic acids with insoluble lead salts such as phosphates.

A few preliminary experiments indicated that the amounts of inorganic acids were not likely to be large. For this the technique of Richardson (1934) was used, with the introduction of a photometric determination of the end-point using a Spekker absorptiometer (Wood, 1950). Results showed that the amounts of inorganic acids were less than 5 per cent. of the total acids, and it is therefore assumed that in *Sedum praealtum* total acidity as measured by lead precipitation is due to organic acids only.

dl-Malic and citric acids. The method used was that of Pucher, Vickery, *et al.* (1934, 1941) and the blue colour of the malic compound was determined in a Spekker absorptiometer using an Ilford yellow-green filter, number 605. *l*-malic acid was also determined by the polarimetric method of Krebs and Eggleston (1943) employing a correction for the interference of *iso*citric acid. The two methods agreed within 10 per cent., indicating that malic acid in *Sedum praealtum* is present as the *l*-isomer.

Isocitric acid. This was determined by the method of Krebs and Eggleston (1944) using as a source of aconitase either rat heart muscle or bullock heart muscle. The citric acid formed was determined by the method of Pucher, Vickery, *et al.* (1934, 1941) and not according to the Krebs and Eggleston modification.

Total reducing sugars. The solution used was the clear centrifugate obtained after precipitation of the organic acids at a pH of 4 to 5, with basic lead acetate solution. The determination was then carried out by the method of Van der Plank (1936). Figures are quoted as mg. glucose per 100 g. fresh weight of leaves.

Non-fermentable reducing sugars. The method used was that of Harding and Selby (1931), which was carried out on an aliquot of the lead-precipitated sample. This fraction of the total reducing sugars includes the Crassulacean sugar, sedoheptulose discovered by La Forge and Hudson (1917). Values are again quoted as mg. glucose per 100 g. fresh weight.

Sucrose. Sucrose cannot be estimated by acid hydrolysis in *Sedum* because of the conversion of sedoheptulose under these conditions to the non-reducing sedoheptulosan (Bennet-Clark, 1933). Hydrolysis with invertase (a solid preparation from G. T. Gurr) was therefore substituted. The reducing sugars formed were then estimated as before.

Starch. The method used is based on that of the Association of Official and Agricultural Chemists, 1945. Two lots of 20 g. of leaves were ground and quickly dropped into boiling alcohol to give a final alcohol concentration of 70 per cent. Heating under reflux was continued for 3 hours; the mixture was then cooled, and the liquid decanted through starch-free paper. Fresh 70 per cent. alcohol was

added, and the precipitate re-heated, cooled, and filtered through the same paper. The precipitate was dried, and the paper added to it in the same flask. The starch was gelatinized by boiling with water for 20 minutes, and after cooling acetate buffer (pH 4·8) was added. One flask serves as a control, while 5 ml. of takadiastase solution (1·5 g. of a Parke-Davis sample adsorbed on talc, in 25 ml. water) is added to the other. The flasks are incubated for 3 days at 25° C. with the addition of toluene to prevent infection, and at the end of this time the samples are filtered and made to volume. The reducing sugar present is then estimated as before. Starch figures are quoted as mg. of glucose per 100 g. fresh weight.

Artificial gas mixtures. These were made up from laboratory air and cylinder carbon dioxide in 10-litre bottles, and kept over saturated sodium sulphate solution.

Leaves (usually 100 g.) were placed in litre flasks of known volume, each fitted with a capillary entry and exit tube, which could be closed by a screw clip. Each flask had 2–3 litres of gas passed through before being sealed. Tests indicated that this resulted in adequate removal of the original air. 10-ml. aliquots were withdrawn at intervals, and the carbon dioxide and oxygen content determined in a Haldane apparatus.

Cylinder nitrogen, when required free of oxygen, was passed over hot copper gauze.

EXPERIMENTAL RESULTS

1. *The diurnal change in acidity*

Malic, citric, and *iso*citric acids together comprise nearly the entire organic acid content of *Sedum praealtum*. Very small amounts of an α-keto acid have been detected in *Sedum* by the use of the colorimetric method of Lu (1939), and the presence of other acids is not precluded.

The present analyses show quite clearly that the typical fluctuations in titratable acidity are caused principally by changes in the malic acid content (Fig. 1 and Table I). Citric acid shows a parallel fluctuation, but this is of a much smaller amplitude. *Iso*citric acid remains approximately constant during both the day and the night, but other work, to be discussed later, indicates that under certain conditions this acid may show fluctuations.

The titratable acidity change gives an approximate measure of the change in malic acid content under a variety of conditions, but a considerable divergence is occasionally found. Care is therefore necessary in ascribing changes in titratable acidity to changes in malic acid.

The diurnal fluctuation in acidity is confirmed as occurring only in the summer months (Bennet-Clark, 1933). In 1947 it started in April and continued until October, showing a maximum effect during September (Figs. 2 and 3). This result is similar to an early one of Bennet-Clark (1933), although the maximum in his experiments was reached in July. This difference is

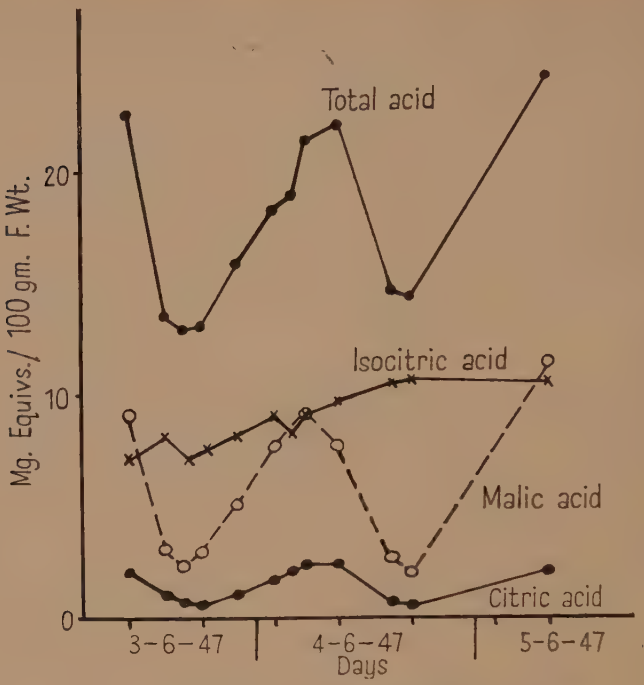


FIG. 1. Diurnal variation in organic acids during June.

TABLE I

Diurnal variation during June 1947

Mg. equiv./100 g. F. Wt. for acids. Mg./100 g. F. Wt. for carbohydrates.

Titrat.	Malic	Citric	*Iso*citric	Total acid	Reducing sugars	Sucrose	Time
9·9	9·1	2·1	7·2	22·5	86	106	10 a.m. 3/6/47
2·0	3·1	1·0	8·2	13·5	316	95	2 p.m. ,,
1·6	2·4	0·7	7·1	12·9	180	95	4 p.m. ,,
1·7	3·0	0·6	7·6	13·0	220	65	6 p.m. ,,
3·1	5·2	1·0	8·2	15·9	200	10	10 p.m. ,,
6·9	7·6	1·8	9·1	18·2	136	43	2 a.m. 4/6/47
7·6	6·7	2·1	8·3	18·8	128	71	4 a.m. ,,
8·2	9·2	2·4	9·1	21·3	128	36	5.30 a.m. ,,
8·4	7·7	2·4	9·7	22·0	204	81	9 a.m. ,,
1·5	2·6	0·6	10·5	14·6	302	104	3 p.m. ,,
2·0	2·0	0·5	10·6	14·2	264	131	5 p.m. ,,
10·9	11·4	2·1	10·5	24·1	192	161	8 a.m. 5/6/47

probably due to seasonal variations from year to year. In particular, tempera ture is known to have a marked effect on the amounts of acids formed in th dark, both in *Sedum* (Bennet-Clark, 1933) and in other members of th Crassulaceae (Wolf, 1938; Pucher, Vickery, *et al.*, 1948). The high early morning acidities found in September are probably due to the low nigh temperatures during that month in 1947.

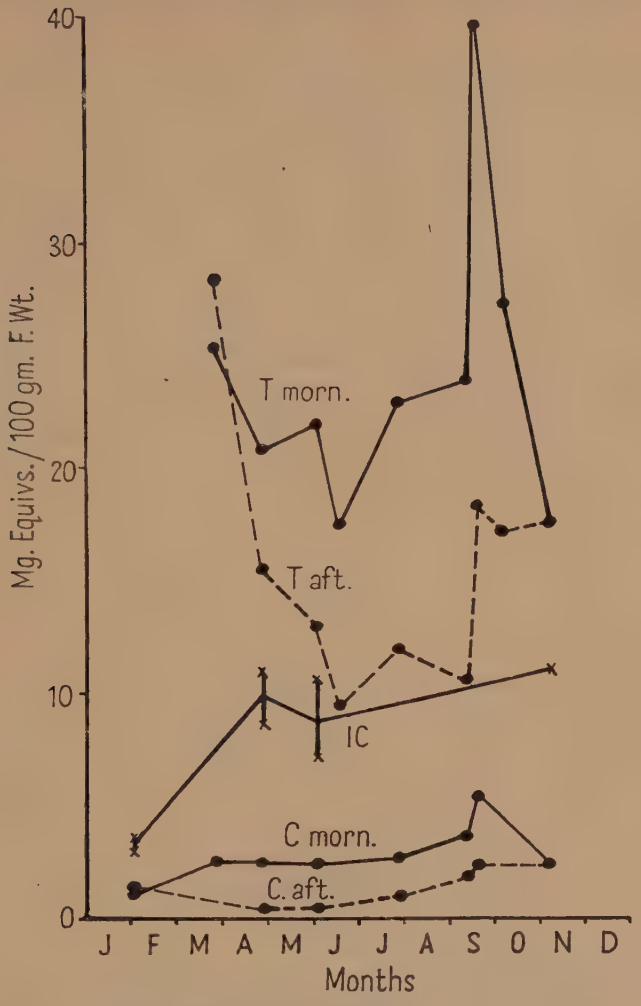


FIG. 2. Early-morning and late-afternoon amounts of total (T), *iso*citric (IC), and citric acids (C) for the different months of 1947.

Among the carbohydrates, sucrose shows no consistent variation with acidity, whereas the total reducing sugars show a constant inverse relationship to the organic acid content. In the early experiments of Bennet-Clark on *Sedum praealtum*, sedoheptulose was shown to account for most of the change in the total reducing sugars. Variations in sedoheptulose (estimated as non-fermentable reducing sugar) are slight, with the exception of the July experiment (Table II).

It seems likely that there is a similar phenomenon in *Sedum*, to that described by Pucher *et al.* (1947, 1948) in *Bryophyllum*, where, according to the age of the leaves, sedoheptulose may account for nearly all the variation in total reducing sugars, or may not vary at all. The remaining carbohydrate,

TABLE II

Diurnal variation between June and December 1947

Figures expressed as in Table I.

Titrat.	Malic	Citric	Total acid	Total reducing sugars	Sedo-hept.	Sucrose	Starch	Time G.M.T.
1·5	—	—	9·5	293	39	19	—	3 p.m. 19/6/47
7·3	—	—	17·5	228	45	50	—	5 a.m. 20/6/47
1·5	5·8	1·0	12·0	387	126	63	—	4 p.m. 29/7/47
9·2	12·8	2·7	23·0	259	63	75	—	6 a.m. 30/7/47
21·8	19·0	3·7	23·9	215	130	87	112	10 a.m. 11/9/47
1·8	4·5	1·9	10·5	473	130	49	429	3.30 p.m. ,,
20·8	20·7	5·4	39·8	265	130	63	111	11 a.m. 15/9/47
1·3	4·1	2·4	18·4	470	111	51	509	5 p.m. ,,
11·4	—	—	27·3	600	—	—	1·0	10 a.m. 7/10/47
2·4	—	—	17·3	613	—	—	182·0	5.30 p.m. ,,

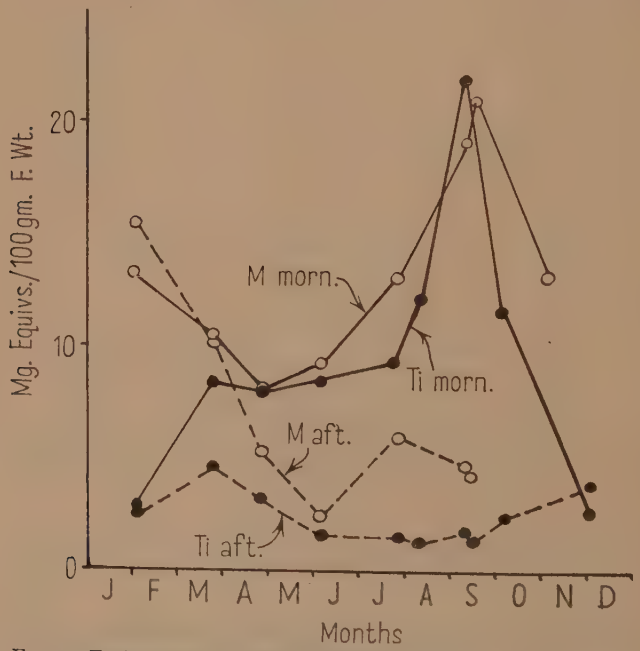


FIG. 3. Early-morning and late-afternoon amounts of titratable (Ti) and malic acid (M) for the different months of 1947.

starch, shows a variation of approximately the same magnitude, but in th opposite sense to that of the malic acid.

2. *The role of carbon dioxide*

The importance of carbon dioxide in acidification has been stressed b Thomas (1949) and is confirmed by the results shown in Table III, whic show the effect of placing leaves in the dark at various temperatures, both i air and in air enriched with carbon dioxide.

TABLE III

Leaves in the dark in air, and in air+CO_2 mixtures

Results per 100 g. F. Wt. of leaves. Figures in brackets are mg. mols; those for starch are expressed as glucose.

Mg. equivs. titrat. acid	ml. at N.T.P. CO_2	O_2	Mg. starch	Time, &c.
		Temp. 5° C.–6° C.		
+5·2 (+2·6)	+3·10 (+0·138)	−38·9 (−1·736)	—	1/6/49. 5 p.m. for 20 hrs in air
+4·46 (+2·23)	+3·2 (+0·142)	−29·2 (−1·304)	—	
+3·18 (+1·59)	−29·2 (−1·304)	−17·0 (−0·76)	—	1/6/49. 5 p.m. for 17½ hrs in 7·5% CO_2/air
+2·88 (+1·44)	−36·1 (−1·608)	−18·2 (−0·813)	—	
+6·09 (+3·05)	−34·7 (−1·55)	−25·9 (−1·157)	—	1/6/49. 5 p.m. for 24½ hrs in 7·5% CO_2/air
+5·07 (+2·54)	−28·5 (−1·27)	−54·0 (−2·41)	—	
+4·32 (+2·16)	−2·23 (+0·1)	−73·0 (−3·26)	−387 (−2·15)	23/6/49. 5 p.m. for 26 hrs in air
+4·42 (+2·21)	+5·85 (+0·271)	−66·5 (−2·97)		
+7·93 (+3·97)	—	—	−494 (−2·74)	23/6/49. 5 p.m. for 23 hrs in 7·4% CO_2/air
+8·33 (+4·17)	−76·3 (−3·41)	−41·0 (−1·83)		
		Temp. 19° C.		
—	+66·49 (+2·97)	−73·4 (−3·28)	—	20/5/49. 4 p.m. for 19 hrs in air
−0·27 (−0·14)	+74·48 (+3·33)	−86·2 (−3·85)	—	
+2·90 (+1·45)	+60·0 (+2·68)	−52·9 (−2·36)	—	26/5/49. 4 p.m. for 14 hrs in 7·3% CO_2/air mixture
+3·76 (+1·88)	+64·8 (+2·89)	−57·8 (−2·58)	—	

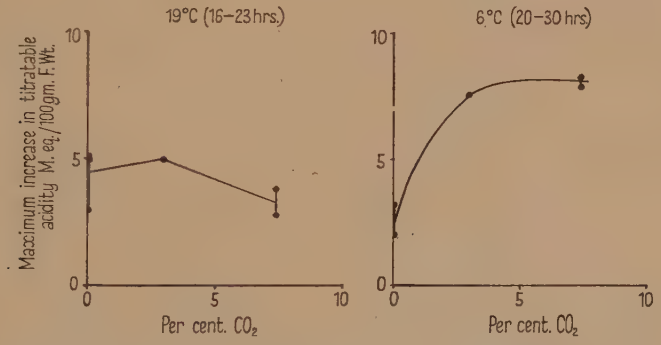


FIG. 4. The effect of CO_2 concentration on acidification of leaves maintained in the dark at low and high temperatures.

Absorption of carbon dioxide from mixtures containing an excess of this gas is marked at low temperatures, and evolution of carbon dioxide from similar leaves in air is negligible: the R.Q. is thus very small. At high temperatures acidification is less marked and the R.Q. is close to unity.

At low temperatures respiration is slow and CO_2 production small, whereas with temperature rise CO_2 output increases. Fig. 4 shows the effect of additional CO_2 on the acid production of leaves maintained in darkness at two different temperatures, 6° C. and 19° C. Maximum acid production occurs at the higher temperature, with an external CO_2 concentration of 0·03 per cent. (air), whereas at 6° C. maximum acidification does not occur until the CO_2 concentration reaches a higher value. This suggests that

Sedum leaves utilize the CO_2 produced in respiration for acid production. A low temperatures acid production is limited by the small amounts of CC available.

Support for the idea that respiratory CO_2 is used in acid formation come from experiments in which CO_2-free air was passed through single leaves by means of the apparatus shown in Fig. 5. It was hoped in this manner t

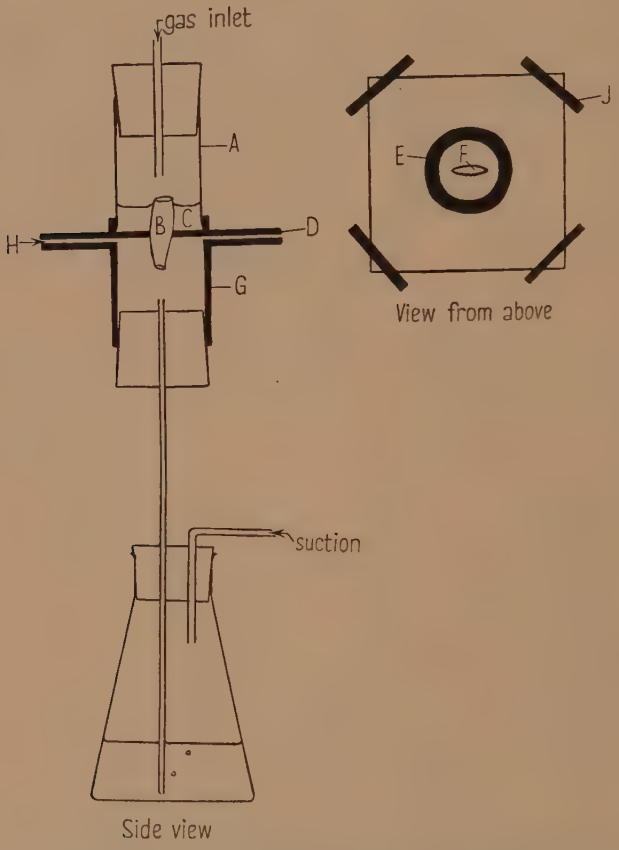


FIG. 5. Apparatus for passing CO_2-free air through a single leaf. A, glass tube; B, leaf held in position by rubber grease; C, layer of water; D, brass plate; E, brass ring soldered to D, into which fits the tube A sealed on with rubber grease; F, slit to hold leaf; G, brass cylinder fused to lower brass plate; H, lanoline between two brass plates; J, screw clip holding two brass plates together.

remove the carbon dioxide evolved in respiration as it was formed. Th effects of this treatment were compared with the behaviour of similar leave left in air in the dark (see Table IV).

The leaves tend to produce less acid under the experimental condition but the results do not show complete inhibition of acid production. There ar probably two reasons for this: firstly the variation between individual leave

TABLE IV

Leaves darkened in air, and in special apparatus with CO_2 free air passing through at 16° C.

(Values for titratable acidity in mg.equivs/100 g. F. Wt. of leaves)

Original	Darkened in air	Experimental	Conditions
3·64	12·55	6·82	Darkened 16 hrs.
2·74	10·30	9·61	
2·53	12·10	8·71	
3·22	10·97	6·93	
3·80	9·33	6·70	Darkened 16 hrs.
1·67	7·75	6·71	
1·44	7·17	7·53	
1·97	9·96	8·78	
4·35	9·32	4·71	Darkened 16 hrs.
3·09	10·20	—	
3·87	—	—	
4·70	11·70	8·66	Darkened 16 hrs.
		6·68	

and secondly the experimental difficulties of maintaining a stream of air through more than one main channel of the intercellular spaces. This latter condition must mean that in some parts of the leaf the carbon-dioxide partial pressure may remain at a sufficiently high level.

The results do, however, suggest the importance of respiratory carbon dioxide in acidification, and show that this must be very rapidly utilized by the acid-forming enzyme system; for even under conditions of much-reduced carbon dioxide partial pressure, there is still marked acid production.

Thomas (1949) has suggested that, in the light, any CO_2 produced in respiration is preferentially used in photosynthesis, and that as a result acid production is halted. Thomas and Beevers (1949) have shown that leaves given an excess of CO_2 can produce acids even in the light.

Further support for this theory of competition for CO_2 between photosynthesis and the process of acid production comes from the following experiments, where part of a leaf was kept in darkness and the rest illuminated.

The leaf of *Sedum* consists mainly of nearly spherical cells with large air-spaces between them, a structure which should facilitate rapid diffusion of gases. In the darkened portion a relatively high CO_2 partial pressure will occur, while in the light portion this will be low. If free diffusion of this gas from one portion to the other is possible, the behaviour of each part may be affected.

The leaves were darkened by means of a wrapping of aluminium foil, tests having previously established that this did not significantly affect acidification.

The proportion of light to dark parts was varied by adopting different ways of darkening as follows: (*a*) Leaf divided into two down midrib. One half darkened. (*b*) Whole leaf covered with foil. (*c*) Leaf untouched. (*d*) Leaf divided into top and bottom halves—top covered. (*e*) Leaf divided into top and bottom halves—bottom covered. (*f*) Leaf untouched, kept in large dark

cupboard. (*g*) Leaf with a wide central dark strip, dividing it into three equa portions—top, middle, and bottom. (*h*) Leaf with narrow central dark stri (3 mm.). (*i*) Leaf with narrow central light strip (3 mm.). (*j*) Leaf with very wid central light strip (2 cm.), leaving very small amounts dark at top and bottom

Leaves were taken at high acidity (in the early morning) and placed in th light. At the end of the experiment they were analysed in separate portions

The results are seen in Table V. Table V shows that the two portion appear to be unaffected by each other. In experiments where the leaf wa divided into three less acidification is found in the darkened middle zon (*g*) (*h*) when this has a light zone on either side. Cutting of the midrib wa tried to see if any longitudinal translocation was involved, but this had n effect. When a very narrow central dark strip is used (treatment (*h*)) this almost completely deacidified, but there is a possibility here of light scatterin from the accompanying lighted zones. The same criticism applies to th results of treatment (*j*).

TABLE V

Leaves in the light from 7 a.m. to 3 p.m. Treatment shown in brackets after th figures which represent titratable acidity (mg. equiv./100 g. F. Wt.)

	Apical half of leaf			Basal half of leaf			Treatment (*i*
Dark portion	9·01 (*f*)	8·74 (*b*)	9·25 (*d*)	8·13 (*f*)	7·23 (*b*)	7·77 (*e*)	8·10
Light portion	3·06 (*e*)	1·88 (*c*)	—	1·91 (*d*)	1·11 (*c*)	—	2·80

Leaves left in the light from 7 a.m. to 8.30 p.m. (D) indicates portion in the dar

Treatment	Portion of leaf Apex	Middle	Base	Remarks
(*b*)	8·54 (D)	9·30 (D)	8·62 (D)	—
(*c*)	—	0·79	1·02	—
(*g*)	0·64	6·42 (D)	1.18	—
(*h*)	0·49	2·06 (D)	0·98	—
(*g*)	0·95	6·0 (D)	1·19	Midrib cut at bottom dark section
(*g*)	0·72	5·61 (D)	1·23	Midrib cut at top of da section
(*g*)	0·79	5·93 (D)	1·17	Midrib cut at top an bottom of dark section

Leaves left in the light from 10.30 a.m. to 3 p.m.

Treatment	Dark portion	Light portion
(*c*)	—	1·25
(*b*)	10·36	—
(*j*)	6·02	1·25
(*i*)	8·66	1·99

4. *Experiments with leaves kept in continuous darkness at different temperatur*

i. *At temperatures from 16° C. to 25° C.* In these experiments leaves we placed in the dark at the appropriate temperature, at a stage when acidificatio should occur (in the late afternoon). Changes in organic acids, carbohydrate CO_2, and oxygen were all studied on the same material and can therefore

correlated. In the experiments involving gas analysis, leaves were kept in sealed litre flasks.

The results in the figures following are given as the changes, + or —, in mg. mols./100 g. fresh weight of leaves, from the beginning of the experiment. In the tables, figures for mg. mols. are in brackets; other figures represent mg. equivs. for organic acids, mg. for carbohydrates, and ml. at N.T.P. for gases. Titratable acidity where used is calculated as malic acid. The validity of this assumption has already been examined in the section on diurnal variation, and will be discussed again later.

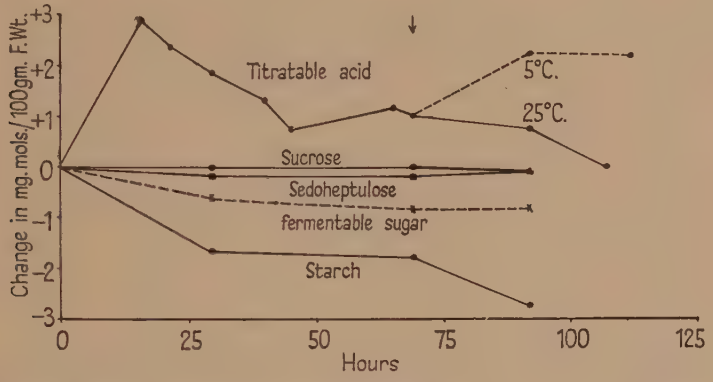


FIG. 6. Effect of placing leaves in the dark in air at 25° C., and changing the temperature to 5° C. at ↓.

In all experiments (see Figs. 6 to 8, Tables VI and VII) the amount of organic acid rises from the initially low level to a maximum, and then begins to fall gradually, the rate of fall being faster at higher temperatures. This formation of acid is accompanied by a loss of starch and an uptake of CO_2. In all these results, following the example of Thomas (1949), CO_2 uptake is calculated on the assumption that in respiration one oxygen molecule absorbed results in the production of one molecule of CO_2, which is then available for acid formation.

TABLE VI

Leaves darkened at 16° C. and at 3° C. in air

Figures in brackets indicate mg. mols. (calculated for titratable acidity asmalic acid).

16° C.		3° C.		
Titrat. acid	Starch	Titrat. acid	Starch	Time
1·93 (0·97)	822·5 (4·59)	1·93 (0·97)	822·5 (4·59)	0 hrs.
10·13 (5·07)	388 (2·16)	11·68 (5.84)	275 (1·53)	20 hrs.
4·89 (2·45)	422 (2·35)	14·00 (7·00)	37·6 (0·21)	66 hrs.
5·34 (2·67)	—	11·47 (5·74)	—	90 hrs.
Changed at 66 hrs. to 3° C.		Changed at 66 hrs. to 16° C.		
6·58 (3·29)	168·6 (0·94)	7·11 (3·56)	193·5 (1·08)	90 hrs.

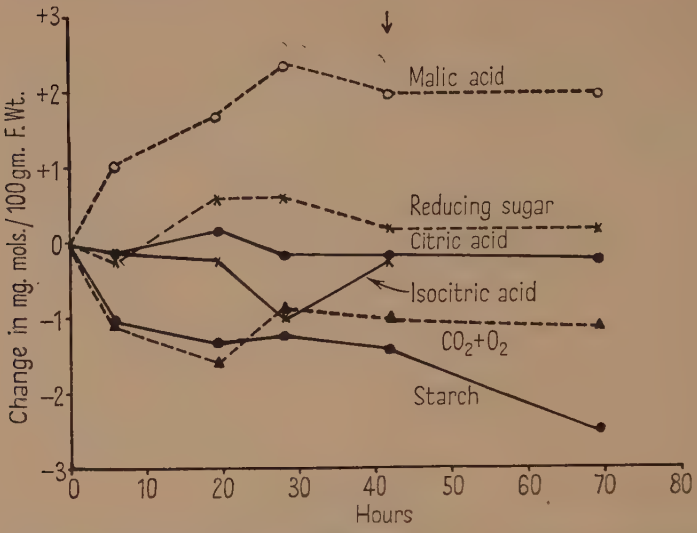


FIG. 7. Leaves maintained in darkness at 19° C. in air. Temperature changed to 4° C. at ↓.

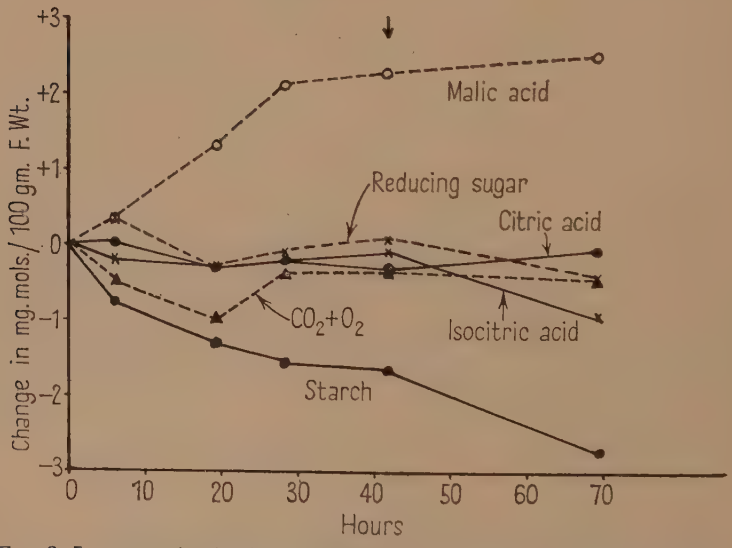


FIG. 8. Leaves maintained in darkness at 19° C. in 3% CO_2/air gas mixture. Temperature changed to 4° C. at ↓. Comparable experiment to that of Fig. 7.

It has already been shown that respiratory CO_2 is sufficient at high temperatures to cause maximum acidification. This is again emphasized by Figs. 7 and 8. In the experiment of Fig. 7 leaves were darkened in air, and in that of Fig. 8 leaves taken at exactly the same time were darkened in air enriched with CO_2. The results show little difference in either the rate or the maximum of acid production. It is seen from these experiments that titratable acidity

does not always provide a reliable guide to the malic acid content. This is because *iso*citric acid also exhibits marked fluctuations, usually in the reverse sense to those of the malic acid. This feature has been noted by Pucher *et al.* (1947, 1948) for *Bryophyllum*, and appears to be characteristic for *Sedum* also.

TABLE VII

Leaves darkened at 20° C. in air, changed to 2° C. at 66 hours

Figures in brackets are mg. mols./100 g. F. Wt.

Titrat.	Total	Malic	Citric	*Iso*citric + rest acid	Total reducing sugars	Starch	CO_2+O_2	Time in hours from start at 5.30 p.m.
1·69	19·28	8·18	1·2	9·90	223·5	826·5	—	0
(0·85)		(4·09)	(0·4)		(1·24)	(4·58)		
3·58	21·9	10·2	4·18	7·52	316	787	(−0·91)	5·5
(1·79)		(5·1)	(1·39)		(1·76)	(4·37)		
6·57	26·4	11·1	4·18	11·12	268	585	(−2·24)	16·5
(3·29)		(5·55)	(1·39)		(1·49)	(3·25)		
7·33	28·5	10·4	2·28	15·82	340	566	(−2·67)	24·5
(367)		(5·2)	(0·76)		(1·89)	(3·14)		
7·14	26·55	10·73	1·88	13·94	281	536	(+1·15)	43
(3·57)		(5·37)	(0·63)		(1·56)	(2·98)		
7·85	27·5	10·45	2·03	15·02	336·5	568	(−0·59)	52·5
(3·93)		(5·23)	(0·68)		(1·87)	(3·15)		
8·04	24·3	7·76	2·51	14·03	287·5	421	(+0·33)	66
(4·02)		(3·88)	(0·84)		(1·60)	(2·34)		
8·08	28·1	11·3	2·12	14·68	368·5	435	(−0·19)	74·5
(4·04)		(5·65)	(0·71)		(2·05)	(2·42)		
8·86	29·8	10·1	2·49	17·21	405	304	(−0·07)	91
(4·43)		(5·05)	(0·83)		(2·25)	(1·69)		

After an approximately steady state of acid loss had been reached, leaves were transferred to a lower temperature, varying from 2° C. to 5° C. It was found that this resulted in a further increase, sometimes small, in the production of organic acids which was accompanied by a drop in the starch content, and in the evolution of CO_2. This result supports the findings of Bennet-Clark (1933), Wolf (1938), Pucher *et al.* (1948), and Somers (1951) that the equilibrium amounts of acid formed are dependent upon temperature. More acid is formed at a low temperature than at a high one.

ii. *Temperatures from 3° C. to 6° C.* The leaves in these experiments were kept in the dark as before, except that the temperature was maintained at about 5° C.

The results are shown in Table VI and in Figs. 9–12. A complicating factor is encountered in these experiments, which is that, at low temperatures, as already mentioned, insufficient carbon dioxide is produced in respiration to give maximum acidification. In the experiments of Table VI and Fig. 9, where gas changes were not measured, the leaves had an inexhaustible supply of air (containing 0·03 per cent. CO_2). But in the experiments shown in Figs. 10 and 11 the leaves were in sealed flasks and had, therefore, no outside source of CO_2.

This difference in availability of CO_2 for acid production has had a great effect on the acidification behaviour. In Table VI and Fig. 9 an early maximum of acidity is found, followed by a very slow fall with time. In Figs. 10

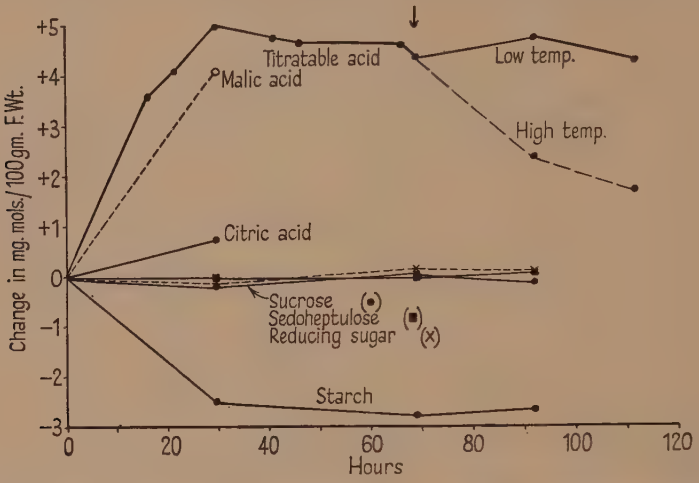


FIG. 9. Leaves maintained in darkness at 5° C. in air. Temperature changed to 25° C. at ↓; the black line for titratable acidity shows the value for leaves kept at the original low temperature for the complete experiment.

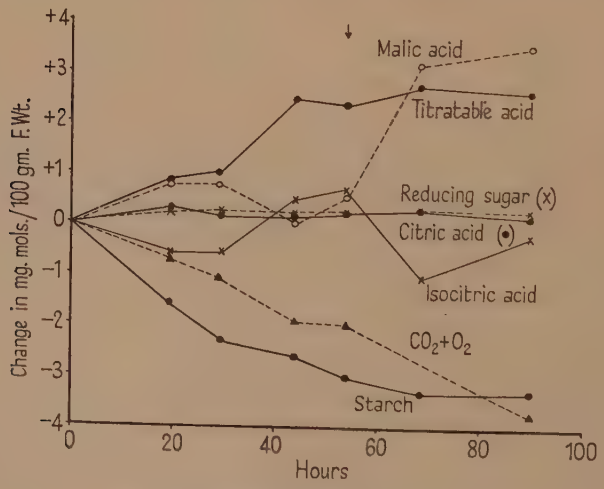


FIG. 10. Leaves maintained in darkness at 5° C. in air. Temperature changed to 18° C. at ↓.

and 11 acid production is at first very slow, being presumably limited by the small amounts of CO_2 produced in respiration at low temperatures. Fig. 1 represents the result of an experiment exactly equivalent to that of Fig. 11 except that instead of air, air enriched with CO_2 was used. This results in more rapid initial acidification.

Acid formation in these experiments, as in those at higher temperatures, is accompanied by starch loss. Malic and *iso*citric acids again exhibit marked interconvertibility. After the first maximum of acid production had been

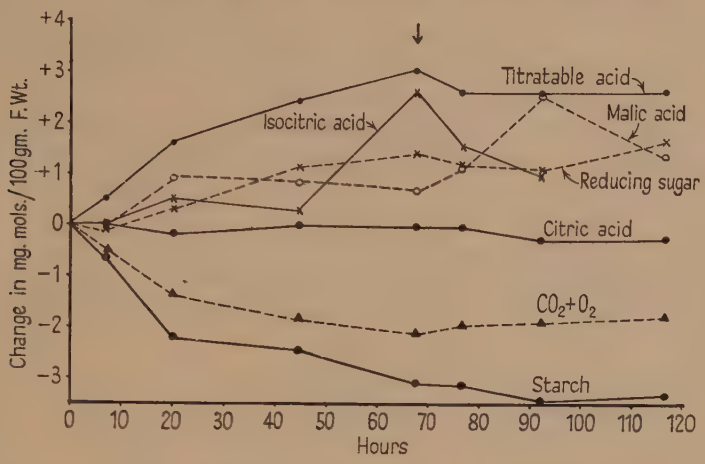


FIG. 11. Leaves maintained in darkness at 6° C. in air. Temperature changed to 15° C. at ↓.

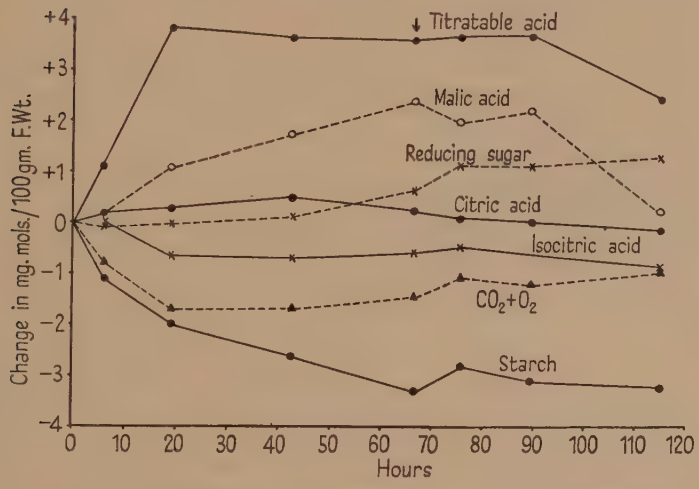


FIG. 12. Leaves maintained in darkness at 6° C. in air enriched with 3% CO_2. Temperature changed to 15° C. at ↓. Comparable experiment to that shown in Fig. 11.

reached, leaves were transferred to a higher temperature. This caused a rapid drop in acidity in those experiments where CO_2 was not limiting (Figs. 9 and 12; Table VI), which was accompanied by a tendency for starch to increase and for CO_2 to be evolved.

In those experiments where OC_2 was limiting (Figs. 10 and 11) transfer of leaves to a higher temperature had the unexpected result of increasing the

malic acid content. This can be explained on the assumption that increas in temperature causes an increase in respiration and hence in CO_2 production This CO_2 is then available for absorption to form malic acid.

In the experiment of Fig. 11 the acid level remains approximately constant but *iso*citric acid is converted to malic acid, a change apparently not involvin external CO_2. There is an indication of a similar effect in Fig. 10, but this i masked by the stronger effect of additional CO_2.

A similar interchange of malic and *iso*citric acid is noticeable in Fig. 12 which again is unaccompanied by any significant alteration in the CO_2 output

DISCUSSION

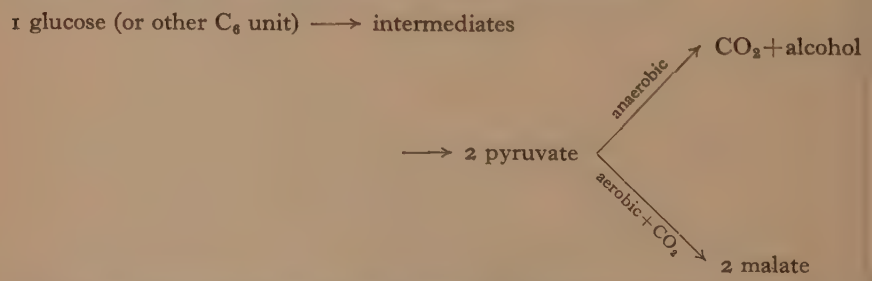
The series of results given in the foregoing sections can be satisfactorily explained on the basis of the scheme suggested by Thomas (1949).

$$\text{Carbohydrate} \downarrow \text{Products of glycolysis} \underset{-CO_2}{\overset{+CO_2}{\rightleftharpoons}} \text{Vegetable acids.}$$

It is probable that interconversions between the various carbohydrates of th leaf are occurring continuously, the net result being that two fractions o the total carbohydrate, starch, and reducing sugars disappear during aci formation.

The loss of reducing sugars is small compared with the amounts of acid formed and may be due either to sedoheptulose (non-fermentable) or t fermentable reducing sugars, depending on the physiological condition of th leaf. Loss of starch always accounts for the major part of the carbohydrat which disappears during acid formation.

If glycolysis and carbon-dioxide fixation reactions are assumed to operat in these plants, the following scheme may be considered:



On this basis, the loss of one carbohydrate molecule (C_6) should result in th formation of two molecules of malate (C_4) with an accompanying uptake two carbon-dioxide molecules (C_1). On a molecular basis the ratio carbohydra loss:organic acid gain during acid formation would be 0·5:1·0, providi that there is an external source of CO_2 available. If this latter condition not fulfilled, part of the carbohydrate may be converted into the carbon dioxi

required. The ratio will then become 0·67 : 1·0, and one C_6 unit (carbohydrate) will produce 1·5 C_4 units (malate).

Under conditions of rapid acidification such as are considered here, it has been shown in the preceding sections that there is little or no evolution of CO_2 from the leaves. It has therefore been assumed that the carbohydrate lost is converted in its entirety to organic acids.

TABLE VIII

Ratios of CO_2, carbohydrates, and organic acids exchanges during the phase of rapid acidification in air. (Calculated on a basis of mg. mols.)

CO_2 (+O_2)	Carbohydrates	Acid	Conditions
—	−0·61 (total carb.)	+1 (malic+citric)	12/8/47. Fig. 9. Darkened 29 hrs. at 5° C. Air not restricted.
—	−0·63 (starch)	+1 (titrat.)	5/7/48. Table VI. Darkened 20 hrs. at 3° C. Air not restricted.
−1·08	−1·52 (total)	+1 (malic+citric +*iso*citric)	18/9/50. Fig. 11. Darkened 20 hrs. at 6° C. Air restricted.
−0·67	−1·29 (total)	+1 (malic+citric)	16/8/50. Fig. 10. Darkened 20 hrs. at 5° C. Air restricted.
−0·61 −0·52	—	+1 +1 (titrat.)	1/6/49. Table III. Darkened 20 hrs. at 5° C. Air restricted.
−1·46 −1·22	−0·99 −0·97 (starch)	+1 +1 (titrat.)	23/6/49. Table III. Darkened 26 hrs. at 6° C. Air restricted.
—	−0·59 (starch)	+1 (titrat.)	5/7/48. Table VI. Darkened 20 hrs. at 16° C. Air not restricted.
−0·77	−1·19 (total)	+1 (malic+citric)	4/9/50. Fig. 7. Darkened 19 hrs. at 19° C. Air restricted.
−1·83	−0·54 (total)	+1 (malic+citric)	24/7/50. Table VII. Darkened 24 hrs. at 20° C. Air restricted.
—	−1·3 (total)	+1 (titrat.)	12/8/47. Fig. 6. Darkened 29 hrs. at 25° C. Air not restricted.

TABLE IX

Ratios of CO_2, carbohydrates, and organic acids during the phase of rapid acidification in CO_2—enriched atmosphere

CO_2 (+O_2)	Carbohydrates	Acid	Conditions
−1·30 −1·73	—	+1 +1 (titrat.)	1/6/49. Table III. Darkened 17½ hrs. at 5° C.
−0·89 −1·45	—	+1 +1 (titrat.)	1/6/49. Table III. Darkened 24 hrs. at 5° C.
−1·29	−0·67 (starch)	+1 (titrat.)	23/6/49. Table III. Darkened 23 hrs. at 6° C.
−0·76	−1·07 (total)	+1 (malic+citric)	18/9/50. Fig. 12. Darkened 42 hrs. at 6° C.
−0·92	−0·77 (starch)	+1 (malic+citric)	4/9/50. Fig. 8. Darkened 19 hrs. at 19° C.

Tables VIII and IX show the figures for this molecular ratio which has been calculated from the results already given. The variation is unfortunately large, but it is notable that in the case of the ratio carbohydrate loss : acid gain, the

figure is below unity in a majority of cases (8 out of 13). The lowest figure for this ratio are shown in experiments where the supply of CO_2 was no restricted (i.e. when the leaves had access either to an excess of normal air or to air enriched with carbon dioxide).

It has been shown in the preceding sections that *Sedum* leaves are able t utilize even the small quantities of CO_2 present in normal air (0·03 per cent. for acid production. The ratio CO_2 intake:acid production is also shown i Tables VIII and IX, and approximates to unity in a majority of cases, th mean value being 1·10:1·00. These figures are liable to error as they ar calculated on the assumption that normal respiration provides one CO molecule for every O_2 molecule absorbed.

During acidification there is frequently no output of CO_2, and it may b assumed that this is utilized in acid formation as soon as it is produced, b processes involving CO_2-fixation enzymes such as those described by Ven nesland *et al.* (1947, 1951) and Ochoa *et al.* (1950).

The reactions involved in acid disappearance have not been so fully investi gated. Acids have been caused to disappear in darkness by an appropriat temperature change, and under these conditions evolution of CO_2 and tendency for an increase in starch content are noticeable. A similar result i reported for *Bryophyllum* by Pucher *et al.* (1947). This suggests that th reactions outlined above may be reversible, but experiments of Varner an Burrell (1950) using C^{14} indicate that this is not the case.

Little consideration has as yet been given to the organic acids, other tha malic, produced by *Sedum*. Citric acid also exhibits a small diurnal fluctuatio and has been assumed to be formed via reactions of the tricarboxylic aci cycle. *Iso*citric acid shows a more variable behaviour and frequently fluctuate in the opposite sense to malic acid. Such interconversions are not easil explicable on the reactions of the tricarboxylic acid cycle, because of th absence of changes in the carbon dioxide output. When *iso*citric is converte to malic acid an extra evolution of CO_2 would be expected, which is no found to occur. This, therefore, suggests the possibility of a different reactio being concerned.

I wish to thank Professor T. A. Bennet-Clark for most helpful advice an criticism during the course of this work.

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The Development of the Enzyme Complement in Growing Root Cells

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SUMMARY

Fifteen serial sections, each 1·0 mm. in length, have been taken from the tip towards the base of the bean root. On corresponding groups of sections determinations have been made of water content, protein content, and of dipeptidase, glycine oxidase, phosphatase, and invertase activities. The number of cells in each section of the series has also been determined. From these data unit cell values have been calculated which show how each of the quantities involved changes as the cell grows.

It has been shown that four phases are involved in the early development of the cell. In the first, which is traversed in the meristematic zone of the root, the volume increases slightly, the protein content decreases, and the activities of three of the enzymes studied also decrease. In the second, volume, protein content, and all enzyme activities increase considerably. In the third, while the volume enlarges by increase in breadth only, protein content and enzyme activities decrease. In the fourth, growth has ceased and protein content, and enzyme activities continue to decrease.

The significance of these changes is discussed.

INTRODUCTION

IT has been shown that the growth of the cell in intact organs may be accom panied by a considerable increase in the protein content (Blank and Frey Wyssling, 1941; Kopp, 1948; and Brown and Broadbent, 1951). This suggest that in the conditions in which the increase occurs there may be correspondin changes in the activities of different enzyme systems. At the same time variety of observations described by earlier workers also suggest that in th course of growth the metabolic pattern of the cell may change. Berry an Brock (1946) have demonstrated a greater respiratory sensitivity to cyanid in the tip than in more mature regions of the onion root, and Kopp (1948 has calculated that, per unit protein, respiration increases as the cell grow Thus a variety of reports suggest not only that the activities of certain enzym systems may increase but that they may increase at different rates relative t each other and to the total protein of the cell. The present investigation ha been designed to examine this possibility. It may be emphasized that if suc changes occur, they constitute important aspects of the process of cell growt

The general technique that has been used is that developed by Brow and Broadbent (1951). Successive sections have been cut backwards from th apices of bean root, quantitative observations have been made on correspon ing groups, and the results related to the total number of cells in the section When the quantitative value per section is divided by the number of cells

he section a figure is obtained for a representative cell. Since cells are con-inuously being formed in the apex and grow immediately they are formed, he successive sections contain cells in progressively more advanced stages of levelopment. The values for the average cell in each section therefore provide series in the development of a representative cell.

On each group of sections quantitative determinations have been made to ssess the activities of certain enzymes, the protein content, and the volume f an average cell. Enzyme activities have been examined by freezing the ections and then placing them in an appropriate medium in which changes romoted by certain enzymes may be measured. The enzyme systems which ave been selected for study are an invertase, a dipeptidase, an acid phospha-ase, and glycine oxidase. The choice of these systems has not been determined y any assumptions regarding their importance for the process of growth. The choice has been determined by the relative ease of studying the reactions hich are promoted by them. Clearly if activities change at different relative ates it should be possible to demonstrate the situation both with systems hat control the growth process and with others that are not immediately nvolved.

In this investigation the volume of the cell has been estimated from the vater contents of the sections. This is probably a more satisfactory estimate f cell volume than that used by Brown and Broadbent which was based on inear measurements of the sections and which does not provide for changes n the intercellular space system. Further, in this investigation we have used road bean roots, whereas in the earlier peas were used. Broad bean roots have ong growing zones and relatively long sections can therefore be cut from hem. With pea roots the short growing zone imposes the use of short sections vith a consequent relatively high destruction of cells in cutting.

MATERIALS AND METHODS

Three sets of observations are involved in the investigation: (1) on growth, 2) on protein content, and (3) on enzyme activity. Each set, however, depends n basic techniques for growing roots, sectioning roots, and determining the umber of cells in each section. Below, the basic techniques are described irst and then the methods appropriate to each set of observations are discussed n the order indicated.

Basic techniques. The roots are obtained by germinating seeds of broad bean var. 'Aquadulce') in sand in aluminium bowls. The sand is adjusted to a onstant water content and the seeds are thrust into it until they are just overed. Evaporation from the bowls is prevented by a glass sheet which rests n the rim of each vessel. The bowls are incubated at 25° C. for 4 days, fter which seedlings having roots 3·0–3·5 cm. long are selected for experi-nental purposes.

Each set of observations is based on 15 serial sections, each 1·0 mm. long, ut from the tip towards the base of the root. Sections more remote than 5 mm. from the apex have not been used since evidence is available which

indicates that secondary thickening begins intensively at about 15·0 mm from the apex. The sections are cut in a modified hand microtome simila to that described by Brown and Broadbent (1951). The instrument accom modates 10 roots and thus 10 sections from the same region can be cut simul taneously.

The total number of cells in each section of a set is determined by the ce maceration technique of Brown and Rickless (1949). Ten sections are im mersed in a known volume of 5 per cent. chromic acid for 24 hours at roo temperature. Maceration is effected by shaking and then squeezing th material through a fine capillary pipette according to the technique describe by Brown and Broadbent. The density of cells in the suspensions is estimate with a Fuchs–Rosenthal haemocytometer.

Growth measurements. As indicated above, the growth of the cell has bee determined in terms of water content which is taken as a measure of volume The water content of each section is determined as the difference betwee fresh and dry weights. Fifty sections are cut into a weighing bottle which i weighed immediately and again after drying in an oven at 80° C. for 4 hours

The variability of the material with which this investigation has been don has been high, and duplicate determinations of cell numbers on correspondin sections made on different days with different samples of material hav occasionally given results differing by more than 50 per cent., especially i the meristematic region. The variability is no doubt due to slight variation in the temperature during germination, to variations in the degree of con solidation of sand in the bowls, and particularly to variability in the sample of seed used. It has been found difficult to reduce the variability with the larg quantities of material required. All observations have been repeated severa times and the data for cell numbers are the means of 6 sets, and for wate content of 3 sets of determinations.

Protein determinations. For each estimation 150 or 450 sections have bee used. After cutting, the sections are transferred to small specimen tube standing in a mixture of solid carbon dioxide and acetone, which prevent any hydrolysis of the protein that may occur during the time that is occupie in assembling the necessary number of sections. Before further treatment th sample is thawed out in the laboratory, after which it is transferred to a glas mortar and ground to a fine paste which is suspended in glass-distilled wate and made up to standard volume.

Protein nitrogen is estimated as the fraction precipitated by trichloraceti acid. An aliquot of the suspension is withdrawn for the estimation of tota nitrogen. Soluble nitrogen is determined on the remainder after precipitatio in 2·5 per cent. trichloracetic acid. Protein is estimated as the differenc between total and soluble nitrogen.

The nitrogen contents of different fractions have been determined with micro-Kjeldahl technique. A catalyst mixture consisting of powdered seleniun mercuric sulphate, and potassium sulphate (Milton and Waters, 1949) ha been used during the digestion with concentrated sulphuric acid.

It has been found that the protein contents of different samples representing the same region but assembled on different days have differed by as much as 40 per cent. Accordingly four different sets of estimations have been made, and the data given in the next part of this paper represent means calculated from these values.

Estimation of enzyme activities. As indicated above, the general technique adopted in this phase of the investigation has been to kill sections by freezing, and to immerse these intact in a medium in which the rate of a reaction catalysed by some particular enzyme can be measured. In principle this technique has been used by Bottelier, Holter, and Linderstrom-Lang (1943) in an investigation of the distribution of dipeptidase activity along the root of barley. In our experiments sections 1·0 mm. long have been assembled in groups of 10 or 20 placed in small conical flasks standing in a mixture of ice and salt and transferred to a refrigerator in which they are left for 1 hour. During this treatment the liquid in the flasks freezes to a solid mass. Before being used the sections are thawed out in the laboratory. They are washed with glass-distilled water and transferred to another small conical flask to which 2·0 ml. of substrate solution has been added. The flask is then shaken in a water-bath at 37° C. for different periods. After removal from the water-bath the sections are separated from the substrate solution by filtration through a sintered glass filter. The sections are washed on the filter, these washings are added to the filtrate, and this is made up to standard volume.

TABLE I

Mg. $\times 10^{-7}$ *P released per cell from a substrate by acid phosphatase in ground tissue* (*A*) *and frozen but intact sections* (*B*)

Section	*A*	*B*
0–1 mm.	0·96	0·93
1–2 ,,	0·59	0·63
2–3 ,,	0·65	0·61
3–4 ,,	0·86	0·94
4–5 ,,	0·92	1·20

The technique is rapid and convenient and with the acid phosphatase we have shown that it yields values comparable to those given by aqueous suspensions of ground tissue. A sample of serial sections was divided into two groups, on one of which the phosphatase activity was determined after grinding, and on the other of which it was estimated on the untreated sections after freezing. The results are shown in Table I, and it is evident that there is little or no difference between the two sets of results. In this experiment the suspension was prepared by grinding in a phthalate buffer at pH 4·0 in a mortar immersed in ice. The phosphatase activity of the ground tissue was determined by suspending it in a solution of sodium β-glycerophosphate adjusted to pH 4·0 with a phthalate buffer, and after incubating the whole mixture for 4 hours at 37° C. determining the phosphorus released. The action of the enzyme was stopped by plunging the reaction vessel into boiling water and the

phosphorus was determined by the technique described below after precipitation of the proteins with trichloracetic acid.

In the standard procedure the cold treatment kills the tissue and destroys the normal cytoplasmic barriers to solute diffusion. It has been found that treating the tissue at −70° C. by placing it in a mixture of solid carbon dioxide and acetone does not increase enzyme activity. It has further been shown that after the standard treatment mature cells do not plasmolyse. Nevertheless, although the barriers to diffusion characteristic of the living state are not involved, it is still possible that the measured rates may be influenced by the length of the diffusion path or by the presence of membranes around enzyme-carrying granules in the cytoplasm. The fragments are considerably larger than those used by Bottelier, Holter, and Linderstrom-Lang (0·1 mm.) and it is possible that the rates of diffusion within a fragment 1·0 mm. long and 1·3 mm. in diameter may restrict the overall rate of the reaction. This possibility has been examined by comparing rates of reaction given with fragments 0·4 and 1·0 mm. in length. No significant differences with the two fragment sizes have been found when the data are reduced to a unit cell basis. It may be pointed out, however, that even if diffusion within the fragment is restricted by either of the two conditions mentioned above, the error is likely to be a constant one and is not likely to affect the relative significance of the data.

In preliminary experiments the course of the reactions catalysed by each of the enzymes studied was investigated. It was found that with each a substrate concentration could be chosen at which, over the experimental period, the rate of the reaction remained constant with time. This concentration was used in all subsequent experiments.

The results obtained in the observations on enzyme activities are given below in two forms— 'primary' and 'relative values'. The primary values are in terms of titration readings or of grams of product formed. The nature of the primary data are described below in each case in relation to the methods used in determining each activity. The primary data may be reduced to a unit cell basis and in this form all the individual observations of a single series may be compared with each other. For comparing different series with each other it is more convenient to reduce all unit cell data of all series to a common numerical scale. Accordingly a relative value per cell has been calculated by expressing the lowest primary value as 1·0 and making a proportionate adjustment in all the other values of the same series.

Dipeptidase. The dipeptidase examined here is one that hydrolyses alanylglycine and has already been extensively studied by Bottelier, Holter, and Linderstrom-Lang (1943) in the root of barley and by Avery and Linderstrom-Lang (1940) in the *Avena* coleoptile. The reaction has been measured by immersing 20 sections in 2·0 ml. of 0·02 M. alanylglycine and allowing the reaction to proceed for 4 hours in an atmosphere of nitrogen. The hydrolysis of the dipeptide is measured by a formol titration technique. 1·0 ml. of formaldehyde stored over basic magnesium carbonate is added to 5·0 ml. of filtrate

and titrated against 0·0125 N. alkali using phenolphthalein as indicator. In each experiment an aliquot of the original dipeptide solution is titrated and the difference between this and the experimental reading is a measure of the amount of amino-acid liberated. The primary data for this series are derived from the differences between control and experimental titration and they are in terms of ml. of standard alkali.

In these experiments we have followed the practice of Linderstrom-Lang and Holter (1932) in not using buffered solutions. The pH of the solution, which before exposure to the tissue is at about 6·6, does not change by more than 0·1 unit during the course of the reaction, and the original acidity is near the pH optimum of the enzyme (7·4).

The experiments of Bottelier *et al.* were conducted with the tissue thus exposed to air. Bean-root material, however, apparently differs from barley since we found in preliminary experiments that occasionally instead of an increase in the titration value there was a very slight decrease. This suggested that another enzyme system attacks one of the products of hydrolysis. The possibility that one of the amino acids was being consumed in a direct oxidation was explored. It was found that under anaerobic conditions the expected increase in free amino groups was obtained. Further, it was also found that in anaerobic conditions there is no change in solutions of alanine or glycine, but that in air, although alanine is not attacked, glycine is.

Glycine oxidase. Evidence of the presence of this enzyme in bean root tissue was obtained from the observations on the activity of the dipeptidase. As far as we know this enzyme has not previously been reported in plant tissue, but a similar enzyme has been extracted and partially purified from pig kidney by Ratner, Nocito, and Green (1944), who found that the end product of the reaction is glyoxylic acid and that the pH optimum for the enzyme is 8·3. We have found that solutions of glycine incubated with frozen tissue give a positive reaction for glyoxylic acid with tryptophane and that the pH optimum in bean root tissue is about 8·0.

The activity of the enzyme has been estimated by placing 20 sections in the reaction vessel with 2·0 ml. of 0·017 M. glycine solution adjusted to pH 8·0 with phosphate buffer, allowing the reaction to proceed for 4 hours at 37° C., and then determining the change in the quantity of glycine, by the formol titration technique described above. The change in concentration of the amino-acid is given by the difference between a control and an experimental titration and the primary data are therefore in terms of ml. of 0·01 N. alkali.

Phosphatase. It has been found that bean root tissue contains an acid phosphatase the pH optimum for which is 4·0. The activity of this system has been examined by estimating the release of inorganic phosphate from a solution of sodium β-glycerophosphate. Ten sections are placed in the reaction vessel with 2 ml. of 2 per cent. glycerophosphate solution adjusted to pH 4·0 with a phthalate buffer. After the reaction has continued for 4 hours inorganic phosphate is estimated in 1·0 ml. of the filtrate by the molybdenum blue technique of Martland and Robison (1920). The intensity of colour is

measured in a Hilger–Spekker absorptiometer using a red (608) filter. Th quantity of phosphorus is determined from a calibration curve prepare from standard solutions of potassium dihydrogen phosphate. In this cas the primary data are given in terms of mg. of phosphorus released.

Invertase. Root tissue contains a vigorous sucrose-cleaving enzyme, th activity of which we have measured by determining the rate of production o reducing sugar in a sucrose solution. The high sugar content of the tissue however, has necessitated in this instance a modification of the normal pro cedure. The sections are left in water for 1½ hours, during which time the thaw and the sugar diffuses out. After this treatment the group of ten section is washed and transferred to 2 ml. of 2 per cent. sucrose adjusted to pH 6· with a phosphate buffer. The reaction is allowed to proceed for 1 hour, afte which the reducing sugar produced is estimated by the Hagedoorn–Jense technique (Strepkov, 1937). The primary data in this case are given in term of mg. reducing sugar.

In this paper the system the activity of which is measured by the procedur described above is referred to as invertase. Observations, however, on rates o cleavage with different acidities in different regions of the root suggest tha more than one enzyme may be involved in the reaction. It has been found tha whereas with relatively mature tissue the optimum pH is 6·0, with meriste matic tissue it is 4·5. Results obtained with an unbuffered solution, with on adjusted to pH 6·0, and with another adjusted to pH 4·5 are shown in Table II

TABLE II

g. $\times 10^{-10}$ *of reducing sugar produced per cell in an unbuffered solution (A), in on buffered to pH 4·5 (B), and in one buffered to pH 6·0 (C). Each value is the mea of four observations*

Section	A	B	C
0–1 mm.	6·93	13·5	9·2
1–2 ,,	5·20	7·4	6·3
2–3 ,,	8·00	20·9	28·6
3–4 ,,	34·0	25·2	56·0
4–5 ,,	66·4	35·6	105·0
5–6 ,,	83·2	45·4	109·2
6–7 ,,	112·2	56·5	132·1
7–8 ,,	130·2	65·6	167·0
8–9 ,,	88·8	59·3	131·6
9–10 ,,	101·5	57·1	129·9
10–11 ,,	66·8	38·5	84·4
11–12 ,,	103·9	29·5	70·4
12–13 ,,	51·9	16·9	61·2
13–14 ,,	42·4	17·7	52·6
14–15 ,,	29·9	14·4	45·7

The higher rate in the first two sections at the lower pH shown by these da has been observed repeatedly, and has been given with different buffe and when the acidity has been adjusted by the addition of hydrochloric ac to the solution. Moreover, it has been shown that the different reactions i meristematic and mature tissues is not due to the activation in either of

precursor. Thus the difference may be due to different systems being involved in the two regions. It is possible that the cleavage of the sucrose that has been measured cannot be referred to a simple invertase and when this term is used below it is used with this qualification. At the same time it may be pointed out that observations made at one pH tend to emphasize the activity of the enzyme with that optimum, and although there may be some doubt as to the precise character of the enzyme this does not invalidate conclusions that are drawn regarding the development of enzyme systems in general in growing root cells.

In relation to some data that will be discussed in a subsequent paper we wish to point out that the values for invertase obtained with an unbuffered sucrose solution and with one adjusted to pH 6·0 are approximately the same (Table II).

Errors of the activity determinations. Each of the values for activity given in the next section is the mean of four separate estimations each made with a different sample assembled on a different day. As indicated above, the variability in the material we have been using has been high. This aspect of the experimental situation is shown by the data of Table III which represent four separate sets of determinations of invertase activity. The errors in the other groups of activity determinations have been less than they have been in this series.

In spite of the differences between corresponding samples, however, it is evident from Table III that the same general trend is shown within each series and the means therefore undoubtedly indicate the general characteristics of the situation.

TABLE III

Values for invertase activity in four sets of determinations at pH 6·0. Figures are g. $\times 10^{-10}$ *produced per cell*

Section	*a*	*b*	*c*	*d*
0–1 mm.	8·9	7·0	11·7	—
1–2 ,,	6·8	4·3	7·1	6·9
2–3 ,,	34·4	29·9	23·0	26·9
3–4 ,,	66·2	42·0	56·5	59·4
4–5 ,,	108·3	107·8	83·1	120·9
5–6 ,,	101·9	114·7	95·5	124·8
6–7 ,,	160·4	84·6	113·3	169·9
7–8 ,,	215·8	152·3	143·9	155·9
8–9 ,,	169·4	125·1	120·5	114·4
9–10 ,,	164·7	126·0	123·7	105·0
10–11 ,,	75·8	88·4	75·9	97·3
11–12 ,,	74·3	68·4	70·7	68·4
12–13 ,,	48·4	58·2	69·1	69·2
13–14 ,,	46·5	44·3	66·4	53·1
14–15 ,,	44·7	44·2	53·2	42·5

RESULTS

In this section the results of observations on growth, protein content, and the activities of the dipeptidase, glycine oxidase, invertase, and phosphatase

are presented. In each case the values given are the means of several independent determinations.

Growth. The values for the average number of cells in each section and for the average water content per cell are given in Table IV and Fig. 1.

TABLE IV

Section	Number of cells per section	Water content per cell (g. $\times 10^{-8}$)
0–1 mm.	45,966	1·31
1–2 ,,	88,303	1·33
2–3 ,,	87,092	1·92
3–4 ,,	55,219	3·38
4–5 ,,	38,047	5·39
5–6 ,,	31,405	7·20
6–7 ,,	22,949	10·50
7–8 ,,	16,681	16·01
8–9 ,,	17,591	16·34
9–10 ,,	16,756	18·13
10–11 ,,	15,832	20·94
11–12 ,,	16,957	20·48
12–13 ,,	17,351	21·31
13–14 ,,	18,078	21·22
14–15 ,,	18,804	20·96

The number of cells increases from the first to the second section and then decreases from the second to the eighth. In the further members of the series the number of cells remains more or less constant, although it shows a tendency to increase from the eleventh to the fifteenth probably as a result of the onset of secondary thickening in this region.

The data for the water content per cell show that the volume increases steadily from the first to about the eleventh section and over this range the increase is about twentyfold. It is significant, however, that the number of cells per section remains more or less constant after about the eighth section. Clearly since the number of cells is constant from the eighth to the eleventh sections but the water content is increasing, it suggests that in this region cell volume is increasing as a result of increasing breadth without change in length. Length increase is restricted to the first eight sections. The data indicate that over the phase of growth represented by the first eight sections the volume increment is due to length and breadth increase, but over the phase represented by the eighth to the eleventh sections it is due to breadth enlargement only. Measurements of the diameters of the sections have shown that the breadth of each increases up to the eleventh section.

The growth data of this investigation may be compared with those of Brown and Broadbent (1951) who worked with peas. These workers found about a twentyfold increase in volume during cell growth. A similar increase has been recorded here with beans. In the earlier investigation, however, a final phase of independent breadth increase was not observed.

It is significant that in this as in the earlier investigation it has been found

that cell volume increases throughout the meristematic region, which in the bean roots of this investigation extends over about the first 3 mm. from the apex.

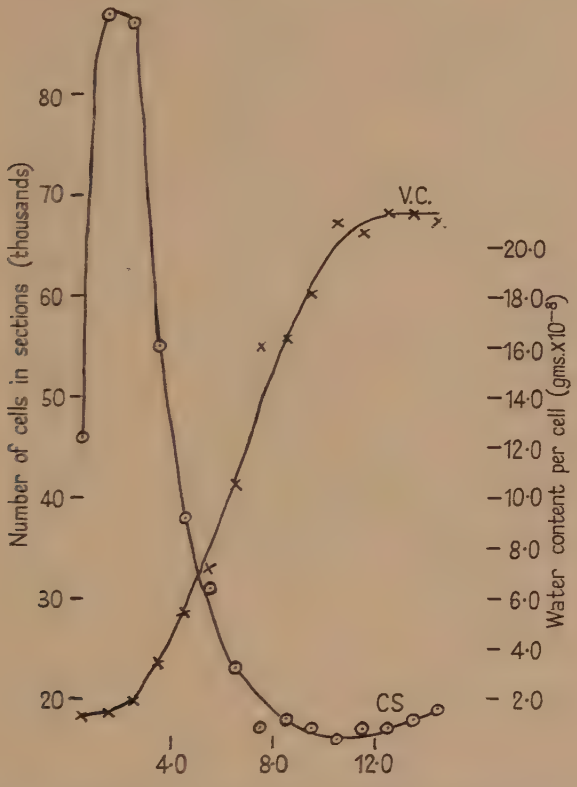

FIG. 1. Numbers of cells in successive sections (CS) and water content of average cell of each section (VC). Abscissae show distances in mm. from root tip.

Protein contents. The protein contents of the successive sections and of the average cell per section are given in Table V and Fig. 2.

Protein nitrogen per section increases from the first to the second, decreases sharply from the second to the sixth, and less steeply from the sixth to the fifteenth.

The protein content of the average cell remains constant or decreases from the first to the third section, increases from the third to the eighth, and decreases from the eighth to the fifteenth section. These data suggest that during the phase of active growth when the cell is increasing in length and breadth there is a two- to threefold increase in the protein content of the cell.

A similar series of observations were made by Brown and Broadbent (1951) with pea roots. Both sets of data show an increase in protein content during growth, although the increase with the broad bean is apparently smaller

TABLE V

Protein-N of successive sections ($g. \times 10^{-5}$/section), PS; and of an average cell of a section ($g. \times 10^{-10}$/cell), PC

Section	PS	PC
0–1 mm.	0·82	1·76
1–2 ,,	1·51	1·70
2–3 ,,	1·37	1·58
3–4 ,,	1·07	1·93
4–5 ,,	0·85	2·23
5–6 ,,	0·64	2·56
6–7 ,,	0·64	2·82
7–8 ,,	0·62	3·71
8–9 ,,	0·57	3·32
9–10 ,,	0·60	3·61
10–11 ,,	0·57	3·51
11–12 ,,	0·59	3·46
12–13 ,,	0·56	3·24
13–14 ,,	0·48	2·65
14–15 ,,	0·52	2·76

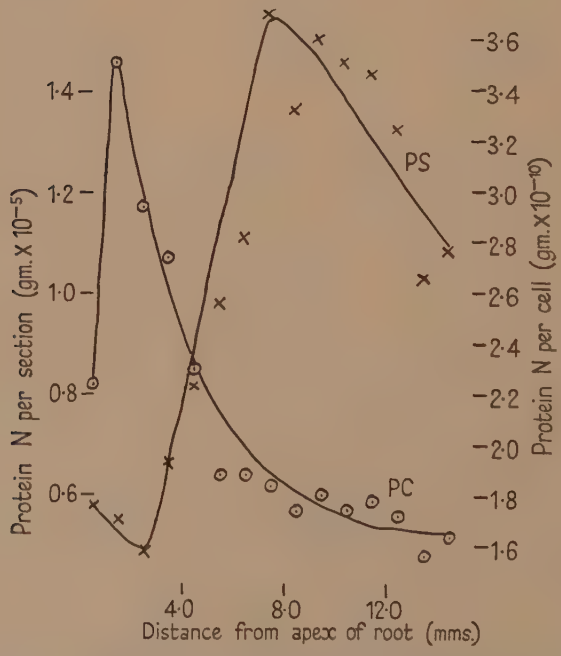

FIG. 2. Protein nitrogen contents of successive sections (PS) and of average cells in sections (PC).

than it is with the pea. Also both the present series of data and those of Brown and Broadbent show a decrease in protein nitrogen after the peak value is reached. The earlier set of data do not show the initial phase of constant or decreasing protein content. This is probably related to the fact that whereas in the pea the meristematic zone is restricted to about the first

millimetre, in the bean roots grown under the conditions of our experiments it extends over about the first 3 mm. Conditions that are evident in the longer meristem may escape detection in the shorter.

The protein data of Fig. 2 are of some significance in relation to the growth data shown in Fig. 1. Increase in volume over about the first 3·0 mm. is evidently limited, and it is over this range that protein content decreases or remains constant. The peak protein content is reached at about 8·0 mm., which is the point at which growth in length ceases.

After 8·0 mm., when protein nitrogen is decreasing, an increase in breadth nevertheless still occurs. The data suggest that breadth increase and length increase may be due to different mechanisms.

Dipeptidase activity. The relative mean values per cell in the successive sections for dipeptidase, glycine oxidase, invertase, and phosphatase activity are given in Table VI. The primary dipeptidase values for the successive sections and for the average cell in each section are given in Fig. 3.

The dipeptidase data given in Fig. 3 show that the activity of this enzyme in the successive sections increases from the first to the third, decreases from the third to the twelfth, and then remains more or less constant. The changes in the series of sections found in this investigation are similar to those recorded by Bottelier, Holter, and Linderstrom-Lang (1943). These workers also found that the peak activity occurs some distance from the tip, although with the barley roots they were using the peak is nearer the apex than it is in bean roots.

In terms of the average cell, dipeptidase activity increases steadily from the
o the eighth section, decreases from this to the twelfth, and then probably
ns approximately constant. From the first to the eighth section there is
a fivefold increase in activity (Table VI).

omparison of these data with those of Fig. 2 shows that while activity is
easing over the first 3 mm. protein content is not, that both reach a peak
the same point (8·0 mm.), and that both decrease after this stage.

Glycine oxidase. The primary data for the successive sections and for the average cell in each section are given in Fig. 4. The section data show that activity increases from the first to the second section, and then decreases steadily from this to the fifteenth. In terms of the average cell, activity decreases from the first to the third section, increases from the third to the eighth, and decreases again from the eighth to the fifteenth. From the third to the eighth section there is about a fourfold increase in activity per cell.

The comparison with the protein data of Fig. 2 shows that, per cell, protein content and glycine oxidase activity decrease from the first to the third sections, from the third to the eighth both increase, and from the eighth to the fifteenth both decrease.

Invertase activity. The primary invertase values for the successive sections and for the average cell of sections are given in Fig. 5. The activity per section increases from the first to the fifth and thereafter decreases steadily to the fifteenth. This result is similar to that reported by Wanner and Leupold

TABLE VI

Relative mean values of activities per cell of phosphatase (P), glycine oxidase (G), dipeptidase (D), and invertase (I)

Section	P	G	D	I
0–1 mm.	1·8	1·3	1·0	1·5
1–2 ,,	1·2	1·0	2·1	1·00
2–3 ,,	1·00	1·0	2·5	4·5
3–4 ,,	1·6	1·5	2·6	8·9
4–5 ,,	2·0	2·0	3·3	16·7
5–6 ,,	1·8	2·6	3·6	17·3
6–7 ,,	2·3	3·6	4·2	21·0
7–8 ,,	3·2	3·9	4·8	26·5
8–9 ,,	3·0	3·3	4·5	20·9
9–10 ,,	2·9	3·1	4·4	20·6
10–11 ,,	2·9	3·2	4·4	13·4
11–12 ,,	2·6	2·8	4·1	11·2
12–13 ,,	2·6	2·4	3·7	9·7
13–14 ,,	2·6	2·1	4·3	8·4
14–15 ,,	2·5	2·0	3·9	7·3

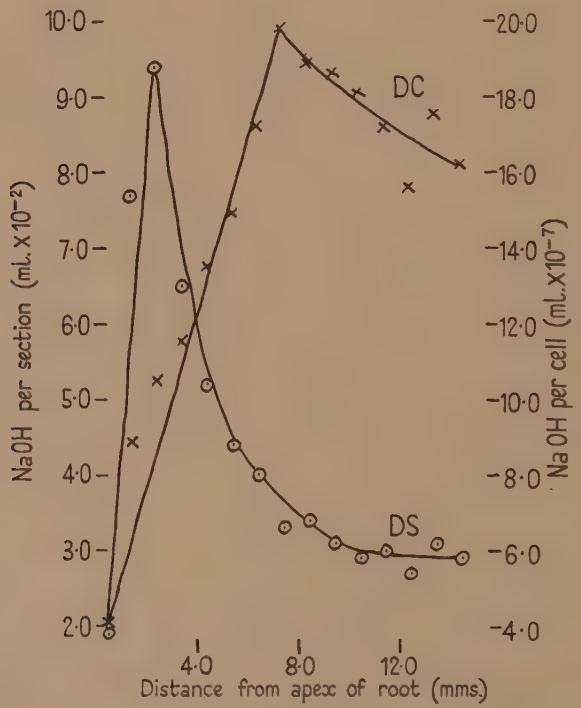

FIG. 3. Primary values for dipeptidase activity per section (DS) and per average cell in each section (DC).

(1947), who investigated invertase activity in different regions of the roo of maize. These workers also found a peak activity at some distance from th apex of the root.

The activity per cell decreases from the first to the second section, increases from the second to the eighth, and then decreases from the eighth to the fifteenth. There is about a twentyfold increase per cell from the second to the eighth section.

A comparison with the protein data of Fig. 2 shows that invertase activity per cell decreases over part of the range over which protein decreases in the

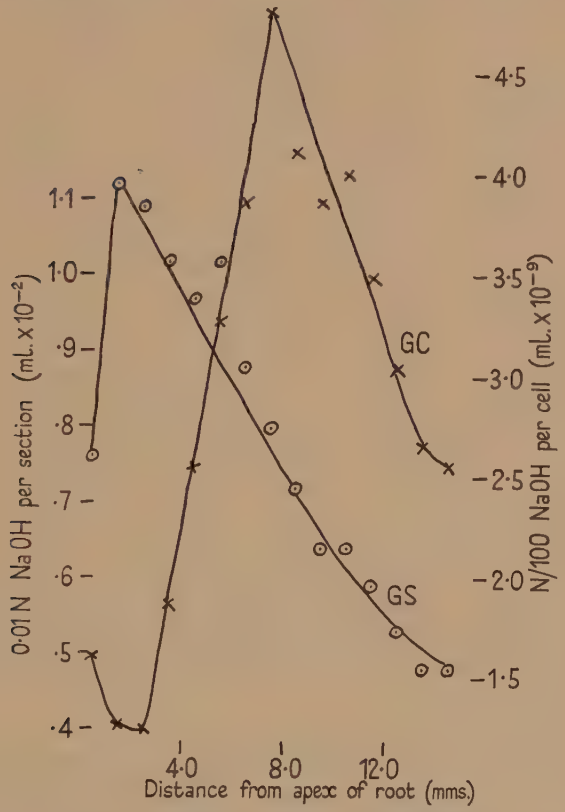

FIG. 4. Primary values for glycine oxidase activity per section (GS) and per average cell in each section (GC).

ip, and whereas invertase activity increases from the second section, protein ontent only increases from the third. Both, however, reach peak values in he eighth section and both thereafter decrease.

Phosphatase. The primary values for the successive sections and for the verage cell of a section are given in Fig. 6. The activity per section increases rom the first to the second section and decreases from the second to the welfth. The activity per cell decreases from the first to the third section, ncreases from the third to the eighth, and then decreases from the eighth o the fifteenth. From the third to the eighth section there is about a fourfold ncrease in activity per cell.

A comparison with the protein data of Fig. 2 shows that, per cell, both activity and protein content decrease from the first to the third section, both increase from the third to the eighth, and both decrease from the eighth to the fifteenth.

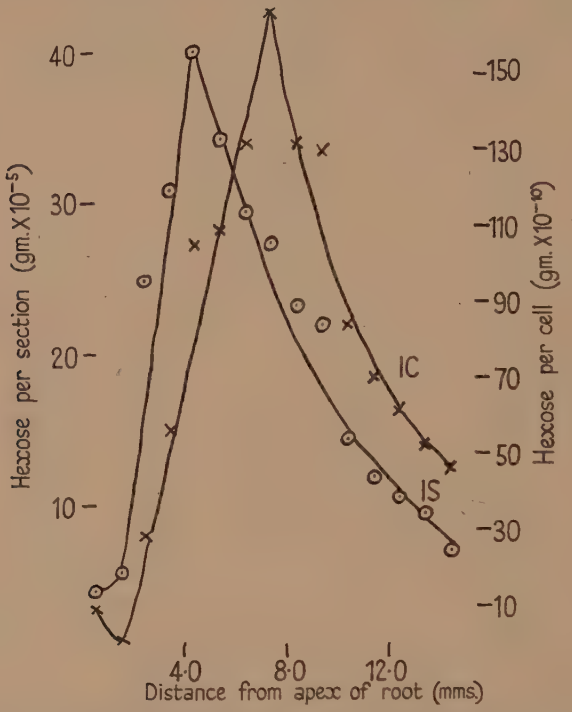

FIG. 5. Primary values for invertase activity per section (IS) and per average cell in each section (IC).

DISCUSSION

The data of this investigation show that there is a close connexion betwee growth, protein content, and enzyme activities of the cell.

In the conditions of this series of experiments four phases are involved i the growth of the cell. In the first, which is traversed within the first 3 mm. c the root and within the meristematic zone, there is a slight but well-define progressive increase in volume, the protein content probably decreases, an the activities of glycine oxidase, phosphatase, and less markedly invertas also decrease. In the second, which corresponds to the change which occu between the third and about the eighth millimetre from the tip, there is large increase in volume, and comparable increases in protein and in th activities of the four enzymes studied. In this second phase, cell growth by increase in length and breadth. In the third phase, which is traverse between the eighth and eleventh millimetres, cell volume continues to enlarg but only as a result of breadth increase. In this phase protein decreases an

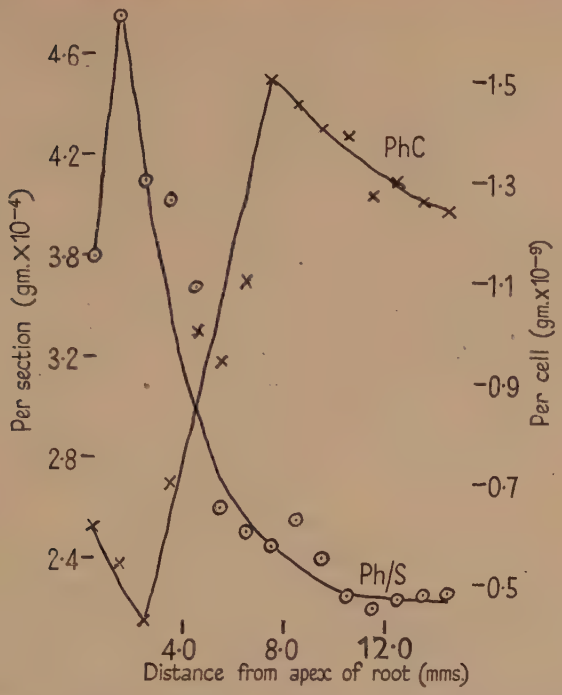

FIG. 6. Primary values for phosphatase activity per section (Ph/S) and per average cell in each section (PhC). Ordinates show weights of P liberated.

he activities of the four enzymes also decrease. In the final phase, which in his series of observations covers the zone extending from the eleventh to he fifteenth millimetre, there is no further change in volume, but the protein evel continues to decrease and the activities of the four enzymes continue o fall.

This complex of changes may be considered in two connexions: the relation f the protein level to enzyme activity and of this to growth.

It is evident that enzyme activity changes intimately with the level of rotein. During the first phase when protein decreases slightly the activities f three of the systems also decrease. During the second, when protein increases, all four activities also increase. During the third and fourth phases, when rotein continues to decrease, so also do the activities of the four enzymes. Clearly the data indicate that the activity level is being determined at least artly by the level of protein. It is evident that to a considerable extent changes n activity are due to corresponding changes in the quantity of enzyme in the ell. At the same time it is also evident that the enzymatic constituents repreent, if not the whole, at least a large proportion of the total protein content. Thus when changes occur in the protein level this necessarily involves correponding changes in the quantity of enzyme and these in turn changes in ctivity.

TABLE VII

Relative enzyme activities per unit protein

Section	Glycine oxidase	Dipeptidase	Phosphatase	Invertase
0–1 mm.	1·6	1·0	1·6	1·4
1–2 ,,	1·0	2·2	1·1	1·0
2–3 ,,	1·0	2·8	1·0	4·9
3–4 ,,	1·6	2·4	1·3	7·8
4–5 ,,	1·9	2·6	1·5	12·7
5–6 ,,	2·2	3·0	1·4	14·3
6–7 ,,	2·3	2·6	1·3	12·6
7–8 ,,	2·2	2·3	1·4	12·1
8–9 ,,	2·1	2·4	1·5	10·7
9–10 ,,	1·8	2·2	1·3	9·7
10–11 ,,	1·9	2·2	1·3	6·5
11–12 ,,	1·7	2·1	1·2	5·5
12–13 ,,	1·6	2·0	1·3	5·1
13–14 ,,	1·7	2·8	1·5	5·4
14–15 ,,	1·6	2·5	1·4	4·5

It is clear, however, that the activity is not being determined simply by tl level of protein. This is shown by the change in dipeptidase activity duri the first phase, which increases while protein is decreasing, and by the differe relative changes in activity of the four enzymes during the whole course development. This aspect of the position is emphasized by the data of Tab VII, in which relative activities per cell have been calculated on a unit prote basis. It is evident that on this basis there is little or no change in phosphatas that from the first or second section to the sixth or seventh there is a twofo increase in glycine oxidase, about a threefold increase in dipeptidase, and fourteen-fold increase in the invertase. After the sixth or seventh sectio there is a slight decrease in the glycine oxidase, little change in the dipeptidas but a considerable decrease in the invertase. These relative changes in activi may be due to a variety of factors. One possibility, however, which it is pr posed to investigate more fully, is suggested by the data of Reith and Morga (1952). It is that during the growth of the cell the structure and compositio of the protein complex changes and that it is as a result of such changes th variations occur in the relative activities of different enzymes. The da of Reith and Morgan suggest the possibility that protein hydrolysates fro the meristem and from mature regions of the root give different chromat graphic patterns. Clearly such differences if they occur may be accompani by changes in the physical state of different components of the prote complex.

It may be noted that the results of this investigation tend to confirm tho of Brown and Wightman (1952), who showed that the meristematic c depends on a flow of metabolites from mature regions. This suggests that t metabolic patterns of cells in the two regions are different. In this investig tion we have shown that during growth the activities of different syste

ıange relative to each other. This necessarily implies corresponding changes ı the intensities of different metabolic processes.

It is possible that the growth of the cell is determined by change in the rotein content as such. It is possible, for instance, that since expansion in- olves a dispersal of the cytoplasm over a greater area, growth cannot occur nless sufficient protein is being continuously synthesized to maintain a onstant thickness in the cytoplasmic layer. Some such structural factor ıay indeed be involved in one or other phase of growth. At the same time is evident that changes in protein and growth do not always correspond. n the second and fourth phases a direct relation between growth and protein ontent might be involved. While rapid growth in the second phase is accom- anied by an accumulation of protein, the cessation of growth in the fourth ıvolves a decrease in protein content. On the other hand, although growth ccurs in the first and third phases, nevertheless in these protein content ecreases. Clearly although protein as such may affect the situation, it is not a etermining factor at all stages of growth.

The data, however, suggest that protein may have an indirect effect on rowth through the determination of the level of enzyme activity. During ıe first phase, when there is clearly little relation between protein and rowth, although the levels of three enzymes decrease, the level of one, the ipeptidase, increases; it is therefore probable that the increase in volume uring this phase is due to an increase in an appropriate enzyme system. In ıe second phase, the increasing volume might be due to an increase in the mount of some particular enzyme system, since during this phase the activi- es of all the systems studied increased. In the third phase, when growth in ength has ceased, the activities of all enzymes decrease. The cessation of ength growth may be attributed to a decrease in some system on which it epends. It is significant, however, that in the third phase, although length rowth has ceased, breadth increase has not. It may be suggested that this is a eflection of a condition in which at the time when length increase ceases readth increase is not limited by the level of the enzymes on which it depends, nd the activities of these may be reduced considerably before this apsect of rowth is affected. During the fourth phase there is no further change in olume and this may be attributed to a probable continued decrease in the vels of growth enzymes.

Brown, Reith, and Robinson (1952) have suggested that the growth of the ell requires to be interpreted in terms of processes that tend to promote it nd of others that tend to arrest it. The nature of these processes is implied y the discussion developed above, but it may be emphasized that the promo- on mechanism involves at least partly the synthesis and activity of enzymes ıvolved in growth, and the arresting mechanism the processes tending to educe the activities of these and other systems.

It is a pleasure to record our thanks to Mr. C. H. Ramsden for technical ssistance.

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The Time Factor in Studies of Growth Inhibition in Excised Organ Sections

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SUMMARY

1. Apprehension over the adequacy of current techniques stimulated a detailed study of the time factor in the arsenate inhibition of growth and respiration in excised stem and root sections of *Pisum sativum*.

2. Growth inhibition by arsenate sets in very slowly, its rate of onset being related to the molar concentration (C) of arsenate by the relation

$$\frac{1}{T_{50}} = KC^{0.64},$$

where T_{50} is the time taken in hours to reduce the growth rate to 50 per cent. of the control and K is a constant. An explanation of the physiological basis of this relationship is attempted.

3. Estimates were made of the final steady growth rate (relative to control) in various arsenate concentrations. The inhibitions calculated from this rate are held to approximate to the true arsenate effect and are shown to be very different from those calculated from 'total growth' measures.

4. Respiration of growing stem sections is not inhibited by the low arsenate concentrations that inhibit growth. Some inhibition is indicated at high concentrations (3×10^{-4} M. and over) but only after 15–20 hours of exposure.

5. Two per cent. sucrose has no effect on the arsenate inhibition of stem growth. Sucrose, however, markedly stimulates respiration in stem sections, but this stimulation is prevented by arsenate.

6. The misinterpretations which may arise as a result of ignoring the time factor in inhibition studies in excised organ sections are discussed and the desirability of constructing complete growth curves in all such studies is stressed.

INTRODUCTION

In recent years investigations into what might be called the 'intimate biochemistry of growth' in the higher plants have made considerable use of inhibitors to which growth and certain metabolic systems (in particular aerobic respiration) show different sensitivities. Most of this work has centred on the extension growth of segments isolated from the growing-zone of *Avena* coleoptiles and etiolated pea epicotyls, the main contributions coming from the laboratories of J. Bonner and of K. V. Thimann in America. Considerations of the relative sensitivities of growth and of respiration to a number of these inhibitors (mono-iodoacetic acid, inorganic arsenic salts, 2:4-dinitrophenol, sodium fluoride, &c.) have resulted in optimistic attempts to identify metabolic systems specifically in control of growth. The writer is not concerned here, however, with the merits, relative or absolute, of these theories, but with the techniques providing much of the data on which they are based.

In the majority of the experiments mentioned above it has been the practic to measure the total growth (increase in length, fresh weight, or dry weigh over a long period after isolation, usually the whole of the subsequent growt period. When inhibitors have been studied the reduction produced in thi total growth has been the direct measure of the growth inhibition. Althoug as the following experiments will show, this is a rather crude estimate, ye observations on concurrent respiration rates have been even less criticall performed. Usually respiration is followed for an hour or so in the earl growth stages and respiration rates quoted as a simple mean value for tha period. Sometimes a similar average rate is given after growth has ceased Rarely are both such mean values recorded.

The inadequacy of such a technique was made very apparent in a recen paper on the selective arsenate inhibition of growth and respiration in *Aven* coleoptile sections (Bonner, 1950). Here growth was measured as total exten sion over a 24-hour period and the arsenate effect recorded as the percentag reduction of this total growth. Respiration (oxygen uptake) was determine only for the third hour after section isolation. It was found that wherea growth was progressively reduced by increasing arsenate strengths from jus below 10^{-5} M. to about 2×10^{-4} M., respiration was not affected over the sam concentration range. Preliminary unpublished work in the writer's laborator on the inhibition of root growth of pea by arsenate had pointed very strongl to a very slow rate of onset of growth inhibition, no marked effect being see in the first 4 to 6 hours except in high concentrations. A similar observatio had already been made by Thimann and Bonner (1949) for arsenite in *Aven* coleoptile sections. It seemed possible therefore that the lack of respirator response in Bonner's experiments might be due to the slowness with which th inhibitor penetrated the tissues and that extension of the respiration observa tions further into the growth period might have revealed an inhibitory effec Furthermore, since much growth obviously takes place during the early perio of arsenate penetration, inhibition of total growth obviously gives a distorte idea of the true effectiveness of arsenate compared with a rapidly penetratin substance.

It should be obvious that what is needed is an accurate comparison of th effects of the inhibitor on the simultaneous *rates* of growth and of respiratio preferably at a time when the inhibitor concentration at the site of its actio has reached the maximum *value* attainable with a given external solutio The reduction in growth *rate* at this point should give a true measure of th inhibitor effect. It was with this in view that the experiments to be describe were undertaken, arsenate being the inhibitor chosen because of its slow actio and obvious suitability for the study of this time factor.

MATERIAL AND METHODS

The material chosen for the bulk of the work consisted of 1-cm. sectio excised from the third internode of 7-day-old etiolated seedlings of *Pisu sativum*. With this material it was possible to obtain a very accurate measure

rowth rates by making use of water uptake as a measure of growth. Sections f pea are much more easily and rapidly dried than cylinders of *Avena* oleoptile and errors in estimation of weight changes are correspondingly maller.

The variety of pea used was 'Meteor' (Sutton and Sons, Ltd., of London). The technique of growing the seedlings and of isolating the sections and the onditions for section growth were precisely as described by Christiansen nd Thimann (1950). All manipulations were carried out in a red light. amples for all experiments were of ten sections. They were floated in a solu- ion of 1 p.p.m. β-indolylacetic acid (IAA) made up in distilled water. The se of buffers has been strictly avoided since previous work has shown that in ome tissues even low concentrations of buffer salts have a marked effect on rowth (Audus, 1949). This was regarded as justifiable, since very little pH rift could be detected during the course of the experiments. Growth took lace in covered Petri dishes in complete darkness at 25° C. in a virtually aturated atmosphere.

Growth measurements were made at 1 to 1½-hour intervals over the first 8 ours of growth after excision and then from 24 to 30 hours on the following ay. Samples were removed from the incubator and sections were lifted out rom the Petri dishes, blotted and weighed to the nearest 0·1 mg. on an air- amped direct reading-balance, and then returned to the solutions. The otal weight of a sample of sections was about 500 mg., this giving a weighing rror of about 0·02 per cent. The errors due to retention of solution on the sur- ace of the sections was not more than 0·5 mg., i.e. an error of 0·1 per cent. Thus a very accurate measure of growth rates was possible even over periods s short as 30 minutes. The samples were out of the liquid for not longer than ½ minutes. The increase in fresh weight is due almost entirely to extension rowth since it has been shown (Christiansen and Thimann, 1950) that the ncrease in length of such sections runs very closely parallel to the increase in resh weight.

In addition to growth measurements a few observations were also made on espiration rates during the growth period using a Warburg constant volume espirometer. Samples of 10 sections, identical with those used in the growth xperiments, were placed in the main chamber of Warburg flasks with 1 ml. f growth medium and again all manipulations were carried out in red light. The medium itself was the same as that used in growth experiments, i.e. p.p.m. IAA in distilled water. No buffers were used in these experiments for he reasons noted above, and again no appreciable drift in pH was observed ver the course of the observations. Oxygen uptake was recorded at 25° C., e. at the temperature of the growth incubator, at 30- to 60-minute intervals n most cases. The inhibitor, sodium arsenate, was placed in the side-arm of he Warburg flask, also in distilled water containing 1 p.p.m. IAA. The ormal respiration drifts were followed for 1½–2 hours in the absence of nhibitor which was then added by tipping. This procedure was necessary order first to establish the basic respiration level for each sample since this

showed a very considerable variation between samples from the same batc (see results later). Thus the inhibitor effect was determined by comparing th subsequent drift of oxygen uptake with that of the control in IAA alone. I each experiment a range of arsenate concentrations was studied. Reading were taken continuously for the first 8 hours after excision. The sections wer then left at 25° C. in the Warburg flasks, which were opened to the atmo sphere and not shaken until the next day, i.e. about 22–24 hours after excision Shaking was then started and the flasks left to equilibrate for 2 hours befor measurements of respiration rate were followed over a further 6-hour perio

GROWTH RESULTS: STEM SECTIONS

General aspects. Fig. 1 shows the growth progress curves calculated a increase in fresh weight and expressed as a percentage of the initial fres weight for 3 samples of sections from the same batch of seedlings in a typica experiment. One characteristic of these sections is the very great uniformit within any one batch of seedlings grown at the same time and under identica conditions. Growth curves of samples from such batches have been found t agree with each other within about 10 per cent. This is strongly contraste with the great differences which are found in the behaviour of differen batches, both from the point of view of the shape of the growth curves an their total extensions, the latter in some cases varying as much as 100 per cen There seems little doubt that conditions of germination of the seeds an growth of the seedlings still involves some unidentified non-uniform factor o factors causing great inter-batch differences. In all the results to be describe direct comparisons have been made only within batches.

All three curves of Fig. 1A show that in the first few hours the growth rat increases with time so that these progress curves are S-shaped. This initia phase of increasing growth rate probably represents the tail end of the expo nential phase of growth of the cells in these sections. It might, on the othe hand, be a recovery from a traumatic inhibition due to excision injury. Wha is clear is that in these studies we are dealing with only the last stages of growth a point which must be borne in mind in any interpretation of results.

The curves show typical effects of IAA and of sucrose. Firstly IAA, a 1 p.p.m., roughly doubles the growth rate, and this effect is maintaine approximately constant throughout the whole of the growth period. Th effect of sucrose in the presence of IAA is very interesting because of the con flicting reports in the literature. Thus Christiansen and Thimann (1950 claimed that they could find no effect of this sugar on total growth extension o such sections, whereas Galston and Hand (1949) observed a considerable in crease of total growth extension in 2 per cent. sucrose although higher con centrations reduced it. In Fig. 1A it will be seen that 2 per cent. sucrose has distinct effect, causing a considerable growth reduction in the first 3 hours, bu subsequently the fall in growth rate, characteristic of the later stages o growth, is not so rapid as in the sections in water. The result is that the fina lengths attained by sections in sucrose are considerably in excess of those i

IAA alone. Although no great number of comparisons of this sort (three separate experiments) have been done in this series of investigations, yet the consistency with which the results have been obtained and the highly uniform behaviour of section samples within any particular batch suggests that the effects are real. As has been shown in the present investigations and also in

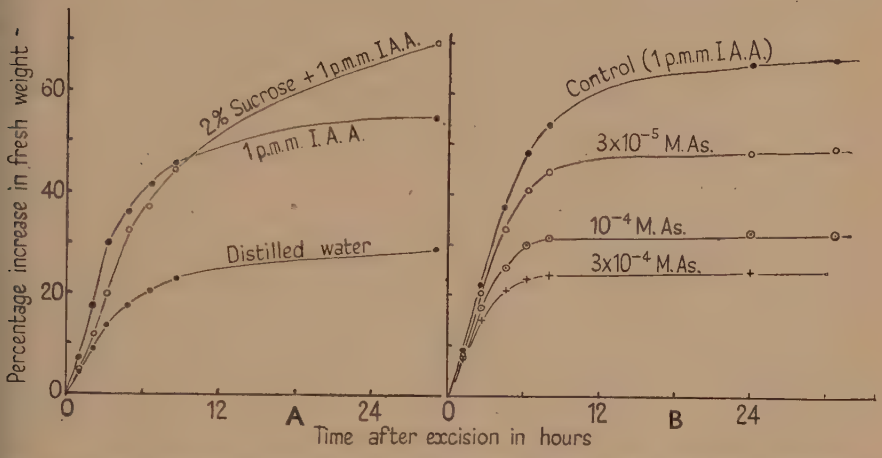

FIG. 1. Growth progress curves for 1-cm. sections excised from 7-day-old etiolated pea stems. A. Typical effects of 1 p.p.m. β-indolylacetic acid and 2% sucrose. B. Effects of a range of sodium arsenate concentrations: all solutions in the series contain 1 p.p.m. β-indolylacetic acid.

those of Galston and Hand, growth curves of samples from any one batch of seedlings agree with each other within 10 per cent. The effects here noted are well outside this variability range. The explanation could be that, in the early stages of growth, osmotic effects of the external sucrose solution might be retarding water uptake, but that subsequent maintenance of a higher growth rate might be due to sucrose utilization as a supplementary carbon substrate, the lack of which is limiting growth in normal sections at this stage. Thus, depending on the time of making total growth comparisons, an inhibition, a stimulation, or no effect on growth could be observed. These results indicate that previous work should be carefully repeated, paying full attention to the possible effect of the time factor.

The effect of arsenate. In the experiments on arsenate, observations have been carried out on up to 6 samples each of 10 sections from a single batch of seedlings. One sample has been used as a control in a solution of 10^{-6} IAA in distilled water. In the remaining samples various amounts of arsenate have been added to give concentrations varying within the range 10^{-2} to 10^{-5} M. Sections are therefore exposed to arsenate during the whole of the growth period subsequent to excision. In Fig. 1B the curves for the increase in fresh weight for 3 such samples and their control has been recorded. It will be observed that the initial growth rates are almost identical, but that the rate relative to the control falls away as growth proceeds and that the progress of

this inhibition is more rapid the higher the concentration of arsenate. In all but the highest arsenate concentration the curves show an initial increase in growth rate, reaching a maximum in the first 2 hours, followed by a rapid fall. The growth actually does not stop in normal sections until after about 40 hours, but it falls to half its initial value after about 7 hours. In the presence of arsenate over this concentration range the growth rate, except at zero time, is lower than the control, the difference being greater the higher the arsenate concentration. Since in these inhibitor studies we are concerned with the effects on growth processes, our best measure of such effects would be the changes induced by the inhibitor in the growth rate relative to that in the control sections. Such changes are shown in Fig. 2, where the growth-rate curves for inhibited sections are redrawn as ratios to the corresponding control curve. It will be seen that in all concentrations arsenate produces an inhibition in growth rate which increases more or less linearly with time in the first 6 to 8 hours of growth. In all three concentrations the curves extrapolate backwards to cut the zero time axis at 100 per cent., indicating that sections in all these inhibiting concentrations have the same initial growth rate. In addition the slopes of the curves increase with rising arsenate concentration. After an initial linear descent these curves eventually flatten out, suggesting that they may become parallel to the time axis towards the end of the growth phase. The most important fact that emerges is that the effect of arsenate is slow and cumulative and does not seem to reach completion in all the above concentrations until the growth period is mainly over. The implications of this in terms of growth-measurement techniques will be considered later.

A much more extended range of arsenate concentrations has in fact been studied, i.e. from 10^{-5} to 10^{-2} M., and ratios of growth rates in arsenate to those of the corresponding controls have been worked out for all experiments. In this way the considerable inter-batch variation in growth behaviour was eliminated. For each sample separate stepped curves have been drawn as in Fig. 2 and smooth curves similarly constructed to fit these measured ratios. From each of these smooth curves a new set of ratios has been read corresponding with 1·5, 3, 6, and 9 hours after excision. All the data have then been brought together in one 3-dimensional diagram. For any one arsenate concentration and any one of the above times from excision, mean values of the ratio from all the relevant smooth curves have been found together with their standard errors. From such data Fig. 3 has been constructed. The two base axes are for arsenate concentrations, which increase from the back of the diagram outwards, and for time from excision increasing from 0 to 12 hours from left to right. The vertical axis represents ratios of growth rate in arsenate to that in control. It should be noted that these results cover only the first 12 hours of growth, over which period the normal growth rate is reduced to about 20 per cent. of its initial value. The open circles represent means and the vertical black lines through the circles twice the standard error of these means. The number of samples involved for each curve is indicated thereon

A number of points emerge from this composite diagram. Firstly, in all high arsenate concentrations the growth rate is reduced to zero during the grand period of growth (i.e. in 10^{-2}, 10^{-3}, and 3×10^{-4} M.). Secondly, in the lower concentrations, there is a suggestion that the curve flattens out towards the axis as time increases, that is, the growth rate is coming to equilibrium at a

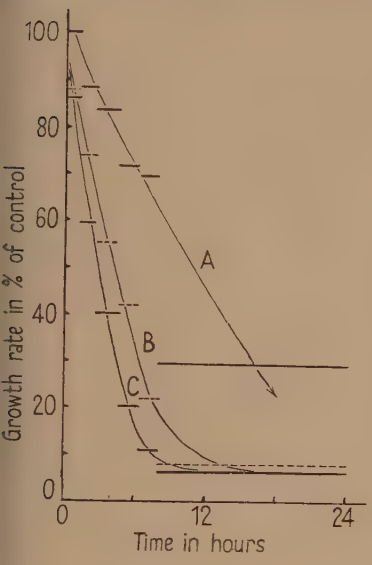

FIG. 2.

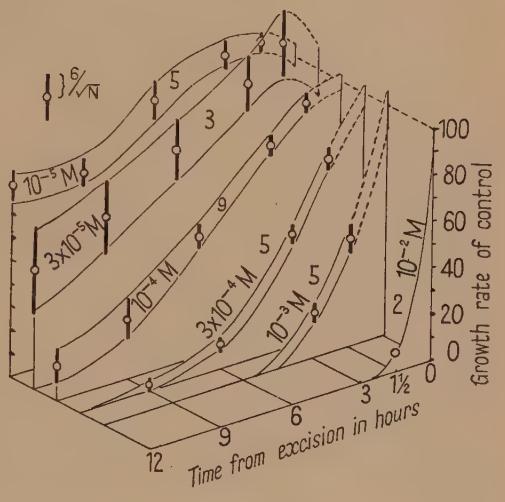

FIG. 3.

FIG. 2. Effects on the growth rates of etiolated pea stem sections of increasing concentrations of sodium arsenate (data of Fig. 1B). A, 3×10^{-5} M.; B, 10^{-4} M., and C, 3×10^{-4} M. arsenate.

FIG. 3. Pooled growth-rate data for pea stem sections showing time-inhibition relationships in all arsenate concentrations studied. The number of samples of sections observed at each concentration is noted on the relevant curve. For further explanation see text.

low value relative to that of the control. This flattening is also suggested in the higher concentration of 3×10^{-4} M. Unfortunately we must still remain in some doubt concerning the reality of this equilibrium point, because of complications and errors introduced by the low growth rates in the controls, although considerable support for it comes from studies of roots, where the grand period of growth is of much longer duration (see later). In any event these low values of growth rate represent an approximation to the true effect of arsenate once it has attained a steady concentration at its site of action in the extending cell. This gives a picture quite different from the one obtained by taking the inhibition of total extension as a measure of arsenate action.

The third point of interest is that in low concentrations (10^{-5} and 3×10^{-5} M.) growth rates over the first 3 hours are stimulated before the inhibition sets in. In 10^{-5} M. this stimulation is statistically significant at the per cent. level; in 3×10^{-5} M. this is not so, although the data suggest that with more experiments it might become so. This stimulation is no doubt

brought about by the very low concentrations of arsenate attained at the growth centres at this early stage and is consistent with the general phenomenon of inhibitor stimulation in such low concentrations. Later, when concentrations rise at the growth centres, this stimulation gives way to an inhibition. By taking growth measurements after 24 hours only, these two opposing effects would neutralize one another, at least partially, and the net effect on total extension is small or nil (see Fig. 4).

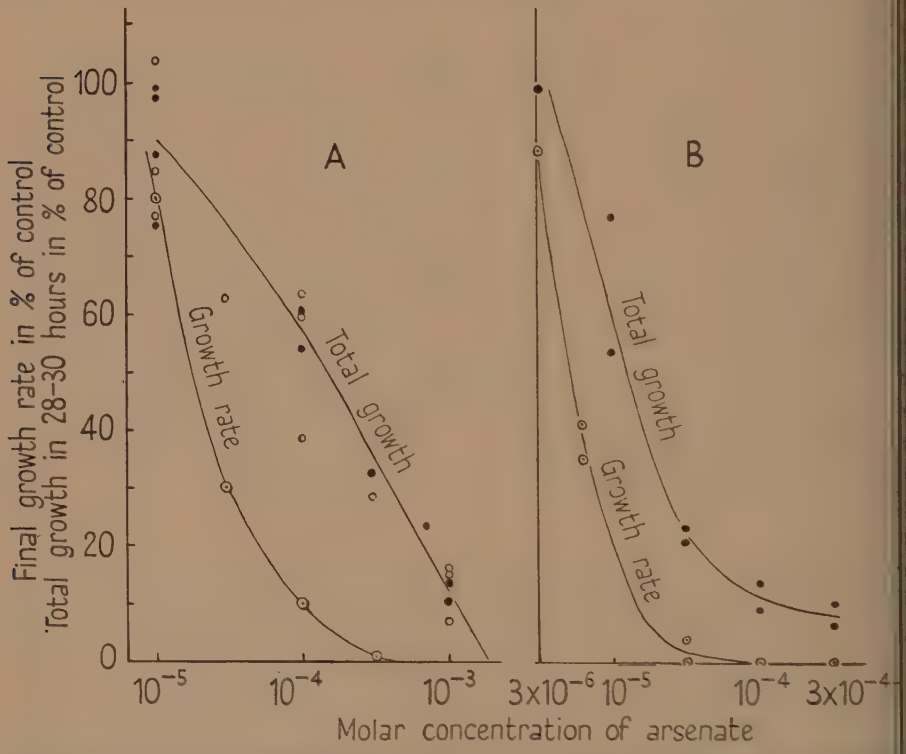

FIG. 4. Growth inhibition-inhibitor concentration curves for sodium arsenate as calculated on the 'total growth' basis of previous workers and also on the final growth-rate basis of the present paper. A. Curves for 1-cm. stem sections of pea (○, sucrose; ●, water). B. Curves for 2-mm. root sections of pea.

It is obvious therefore that the exact relationships between inhibitor concentrations and growth, which have played such an important part in recent theories, are very much dependent on these time effects. Thus growth inhibitions, as measured by total extension, are far too low, since much of the growth was carried out while the inhibitor was gaining access to the growth centres. This discrepancy is well illustrated from the present results in Fig. 4. In graph A the effect of arsenate has been expressed in two ways, one as inhibition of the fresh weight increase during the whole of the growth stage after excision (curve labelled 'Total growth') and the other as the inhibition determined from the growth rates after 12 hours (from the means of Fig. 3).

this being an approximation to the ultimate equilibrium growth rate when arsenate is exerting its maximum effect. In the first of these graphs (Total growth) the inhibition over the arsenate range 10^{-5} to 10^{-3} M. is directly proportional to log. concentration. This is identical with the relationship found by previous workers using the same measure of growth. The 50 per cent. inhibition concentration occurs at about $1{\cdot}4 \times 10^{-4}$ M. The second graph of growth-rate inhibition shows no such linear relationship with the log. concentration but is markedly convex to the concentration axis in addition to being very considerably shifted towards the lower concentration ranges. The concentration for 50 per cent. inhibition of growth rate is $1{\cdot}7 \times 10^{-5}$ M., i.e. about $\frac{1}{8}$ of the former estimate.

From the original growth-rate inhibition data as illustrated in Fig. 2 estimates have been made of the rate of onset of inhibition and its relation to arsenate concentration. To obtain a measure of this rate the time taken for the inhibition to reach 50 per cent. ($= T_{50}$) has been determined for all relevant samples. The reciprocal of this time ($1/T_{50}$) gives a reasonably accurate measure of the rate of inhibition in these early stages where, except for very low concentrations, the inhibition progress curve is virtually linear. In Fig. 5 these calculated rates are plotted against arsenate concentration on a logarithmic scale. The graph shows that there is a reasonably linear relationship between these two variates over the range 3×10^{-5} to 10^{-2} M. At lower concentrations than this (i.e. 10^{-5} M.) the line suddenly plunges downwards, since at that concentration the 50 per cent. inhibition level is not reached during the course of the growth observations. These two variates give on analysis a correlation coefficient $r = 0{\cdot}926$, which is highly significant, and a regression of log. $1/T_{50}$ on log. arsenate concentration (C) equal to 0·64. This means that we can write the relationship of the rate of inhibition onset ($1/T_{50}$) to the concentration of arsenate (C) in the form

$$\frac{1}{T_{50}} = KC^{0{\cdot}64}.$$

From the constants of the regression equation K is found to be approximately equal to 14, where T_{50} is expressed in hours and C in molarity. This relationship has the same form as the Freundlich adsorption isotherm and is closely similar to a relationship found by the writer in previous experiments on the rate of inhibition of growth in attached roots of pea seedlings after external application of low concentrations of 2:4-dichlorophenoxyacetic acid. It suggests that the rate of inactivation of the growth processes is proportional to the concentration of inhibitor adsorbed at some colloidal interface, which is not the growth centre itself. This interface could be in the surface layers of the root at or near the point of entry of the inhibitor. It seems probable that this adsorption takes place rapidly and reaches equilibrium in a very short time after the sections have been placed in the solutions. The rate of movement of arsenate from this adsorbed condition into the cell and thence to the growth centres is slow and proportional to the concentration of

adsorbed arsenate ions. Now the rate of inactivation of growth, i.e. the tim taken for the growth rate to be reduced by a given amount, will depend o the total amount of arsenate which has reached the centre in that time. Sinc this rate of accumulation is proportional to the concentration gradient, th rate of growth inactivation itself will be proportional to the adsorbed con centration. This argument would also hold for 2:4-D inhibition, although c course the equation constants would be different. Such considerations sho the complexity of an apparently simple situation where all components of th growth system are reduced to their simplest form and stress the innate diffi culties of studying the effects of externally applied reactants on such dynamic system as a growing cell.

The effect of sucrose on arsenate inhibition. We have already seen that sucros itself has some slight effect on the growth rate, causing an initial retardatio which is offset later by a slight prolongation of the grand period of growth There is, however, no effect of sucrose on the degree or progress of arsenat inhibition, as can be seen by a comparison of the open circles with the close ones of Fig. 4A (total growth inhibition) and Fig. 5 (rate of onset of inhibition These sucrose values have been calculated as ratios to those of controls i 1 p.p.m. IAA plus 2 per cent. sucrose, so that the sucrose effect on growth i thereby eliminated.

The effect of 2:4-dinitrophenol. 2:4-Dinitrophenol (DNP), in contrast t arsenate, is a rapidly penetrating inhibitor and therefore one or two exper ments were carried out to see whether a time effect could be demonstrate here also. Concentrations which were used were 10^{-3}, 10^{-4}, 10^{-5}, and 10^{-6} M Even in the higher concentrations sections continued to grow after excisio making it quite possible to measure the rates of inactivation of growth centr in these sections. Here, too, the times taken for growth to fall to 50 per cen of the control value were determined from the stepped ratio curves (as i Fig. 2). In 10^{-6} M. 50 per cent. inhibition was never reached. Values o $1/T_{50}$ for the other concentrations were plotted in Fig. 5 (triangles). Althoug we have only three points, they do lie reasonably near a straight line runnin closely parallel with that for arsenate although keeping well above it. In th case also we might postulate that the first stages of inhibiting action is a sorption at some section interface from which it is released to the growt centres at a rate proportional to this adsorbed concentration. The rate movement to the growth centres would of course be much more rapid tha for arsenate (nearly 7 times as fast as calculated from the graphs of Fig. i.e. $K = 100$ approx.).

GROWTH RESULTS: ROOT SECTIONS

It was regretted earlier that arsenate penetration to the growth centr was so slow that it was not possible to say with any certainty that in low co centrations the growth rate of stem sections eventually reached a steady l value relative to control, and it was therefore impossible to estimate accurate what was felt to be the true effect of arsenate once it had attained its maximu

concentration at the growth centre. Fortunately sections excised from the growing zone of pea roots and grown in 0·5 per cent. sucrose have a more extended period of growth, the growth rate 15 hours after excision still being of the order of 40 per cent. of the initial value. In root sections growth also

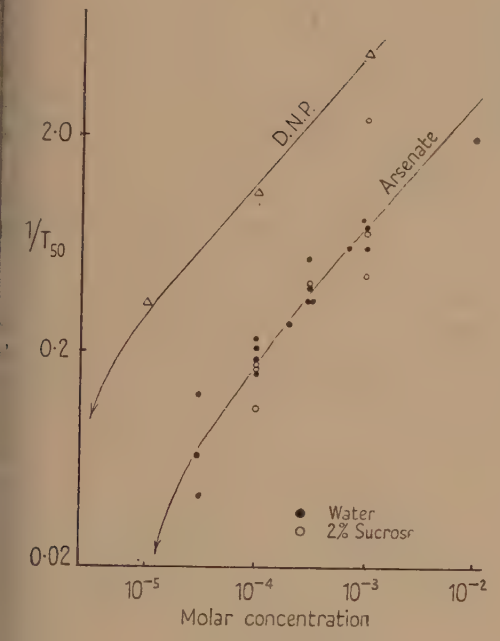

FIG. 5.

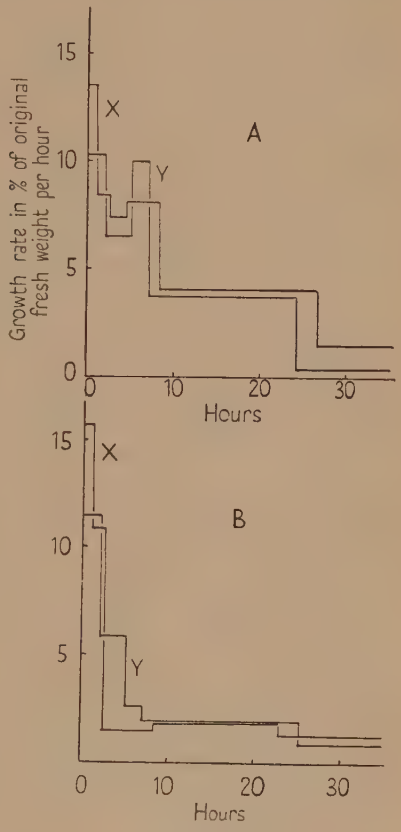

FIG. 6.

FIG. 5. Graphs showing the relationship between rate of inhibition of stem section growth and concentration of inhibitor (sodium arsenate and 2:4-dinitrophenol (DNP)).

FIG. 6. Growth-rate curves for samples of 2-mm. root sections of pea growing in 0·5 per cent. sucrose solution (A) and 0·5 per cent. sucrose with 10^{-5} M. sodium arsenate (B). The two curves marked X are for samples from the same batch of pea roots and grown under the same experimental conditions on the same day. Curves marked Y are for a similar pair of samples studied on another occasion.

continues up to 48 hours or longer, whereas in stem sections it has usually ceased completely after 36 hours. A few experiments have therefore been carried out with such root sections to see how far a steady growth rate may eventually be obtained in weak arsenate solutions.

The technique used will not be described in detail since a full description

will follow in a subsequent publication. The essentials of the method are that 2-mm. sections at a distance of 2 mm. behind the root-tip are cut from 3-day old pea seedlings and are placed in samples of 10 on filter-paper moistened with 0·5 per cent. sucrose. Growth takes place in the dark at 25° C. in a saturated atmosphere and is measured by periodic rapid weighing to the nearest 0·1 mg. Errors due to moisture adhering to sections and during weighing are relatively larger here than with stem sections, but with care these can be reduced to less than 2 per cent. With root sections no precautions have been taken with light conditions and all manipulations have been done in the diffuse light of the laboratory.

The course of growth in typical samples of sections is seen in Fig. 6A where the growth rates are plotted against time from excision. The more extended grand period of growth relative to that of stem sections is clear from these graphs. Note that here there is no indication of a rising growth rate in the early stages of growth as was found in stem sections.

Two growth-rate curves are shown in Fig. 6B for samples of sections from the same batch of roots as those giving the graphs of Fig. 6A but growing instead in 0·5 per cent. sucrose plus 10^{-5} M. arsenate. Here the same two features noted in the stem section results are again apparent. Firstly there is in the first hour, a stimulation of the growth rate. This stimulation is a fairly constant feature of the growth curves obtained in such low concentrations so far and is not confined to this one concentration. Following on this stimulation is a rapid fall of the growth rate to well below that of the control and, what is more, this low growth rate is maintained with little significant change over the remainder of the grand period of growth. It is difficult not to regard this as being maintained by a steady concentration of arsenate at the growth centre as foreshadowed by stem section results. During this period of scarcely altering growth rate in arsenate, normal control growth rate has been steadily falling and so the ratio inhibited growth rate control growth rate has been rising over the same period. This illustrates another aspect of the difficulty of determining from the ratios the true inhibitory effect of arsenate. For an estimate of this ultimate arsenate effect a compromise has been made and the ratio of the average growth rates over the long period 18–24 hours (sections left to grow overnight without weighing) has been taken as a measure of the effect. These ratios have been plotted in Fig. 4B together with similar ratios obtained from the total growth increases after 24 hours. Here, as in the results for stem sections, there is a marked difference in the two curves of arsenate inhibition, the 50 per cent. level coming at $5{\cdot}2 \times 10^{-6}$ M. for the growth rate estimate and $1{\cdot}3 \times 10^{-5}$ M. for the total growth estimate, i.e. the latter concentration being 4 times higher. In stem sections it will be remembered that the discrepancy was much larger, and this can be readily understood when it is remembered that root sections continue to grow for much longer periods. In this way arsenate exerts its effect for a much greater proportion of the grand period of growth and therefore our estimate of inhibition from total growth will be much less distorted by the inclusion of the early penetration period

The growth rate and total growth inhibition estimate curves will therefore fall closer together.

THE EFFECT OF ARSENATE ON THE RESPIRATION OF STEM SECTIONS

Experiments have also been carried out to determine the effect of arsenate on the respiration of stem sections, to check whether the lack of inhibition in Bonner's experiments might be due to slow penetration. As noted earlier, measurements were made on the same samples on two successive days in order to obtain a measure of the respiration drifts during the grand period of growth of the sections. Fig. 7 shows a typical respiration drift curve for sections in 1 p.p.m. IAA. It will be seen that immediately after excision the rate of oxygen uptake falls rapidly, reaching a value about 50–60 per cent. of the initial rate after 7–8 hours and is apparently maintained steady at the rate attained after 18 hours. This drift shows a parallelism with the drift of growth rate, but it is unlikely that this is more than coincidental and that there is any direct causal relationship between them. The cause of this rapid fall in respiration is obscure. It is unlikely to be due to falling concentrations of respiratory substrate since it also occurs when sucrose is provided (see later). It may be due to recovery from traumatic stimulation during sectioning (see Audus, 1935).

One of the unfortunate features of these investigations is the very considerable inter-sample variance of respiration intensity even from the same seedling batch. This differs from the variability in growth response where the large differences were found between batches while samples within batches showed great uniformity. As an example of this respiration variability might be quoted results from 11 samples (10 sections in each) from 3 different seedling batches. Mean respiration intensities were determined for 3 successive half-hour periods after excision and the corresponding standard error determined. For the first half-hour this error represented 5·6 per cent. of the mean, for the second and third half-hours 4·78 and 4·22 per cent. respectively. The higher variability for the initial reading may be due to incomplete equilibration or to recovery of sections from manipulation, and thus a value of about 4 per cent. probably represents the true variability of the material. This high variability means that for such sets of 11 samples no treatment effect giving departures from the mean of less than 2·2 times this standard error (i.e. 9 per cent.) can be detected and established as statistically significant. It is interesting to note in passing that this is the order of effect claimed in the literature for some of the auxin stimulations of respiration, although no direct indications of intrinsic variability were recorded. It is difficult to say how the uniformity of the material used in the present investigations could be increased since every effort was made to ensure constant and reproducible growth conditions.

In view of this large variability it was decided to employ a technique in which the respiration of all samples was followed for the first 1 to 2 hours in the absence of inhibitor in order to establish the normal drift level. Then arsenate was tipped in from the side-arm of the vessel and subsequent drifts

in the inhibitor compared with that in the control. To make this comparison easier all respiration records were recalculated as a percentage of the average respiration before tipping, thus eliminating the inherent variability between samples. The results of a typical experiment of this sort are brought together in Fig. 8A. This shows a family of respiration drift curves for sections in

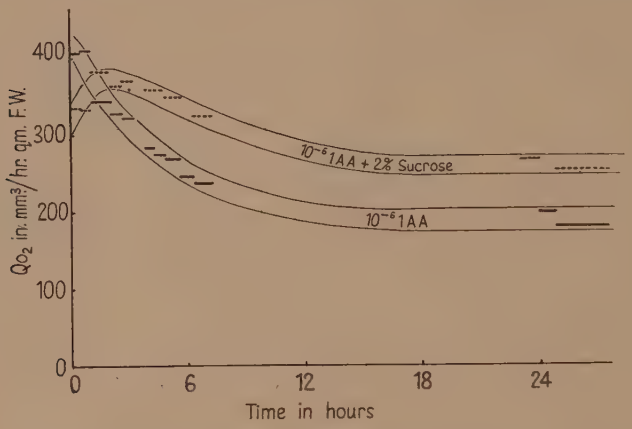

FIG. 7. Respiration drift curves (oxygen uptake) for two comparable samples of pea stem sections growing at 25° C. in 1 p.p.m. β-indolylacetic acid without and with 2% sucrose.

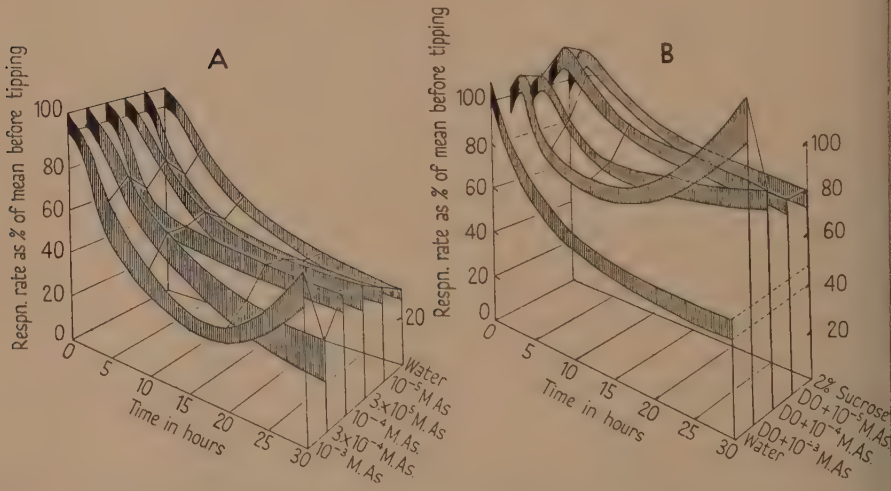

FIG. 8. Families of respiration drift curves for pea stem sections showing the effects o various concentrations of sodium arsenate. For full explanation see text. A. Effects in 1 p.p.m β-indolylacetic acid alone. B. Effects in 1 p.p.m. β-indolylacetic acid + 2% sucrose.

1 p.p.m. IAA (rear curve) and in 1 p.p.m. IAA plus increasing concentrations of arsenate. In each case the thickness of the 'band' represents, as in Fig. 7 the limits within which individual respiration observations fall. The initia black portion of all these curves represents the respiration before tipping. I

ill be seen that even with this elimination of inter-sample variability, no gnificant difference can be established in these drift curves between all con-entrations of arsenate and control sections during the first 10 hours after xcision. By this time in all but the lower concentrations of arsenate the effect n *growth rate* has reached its maximum. Later on, however, i.e. at 15–20 ours, there is a definite suggestion of an inhibition in the respiration in the igher arsenate concentrations (3×10^{-4} and 10^{-3} M.). This inhibition persists or the full 30 hours in 3×10^{-4} M. arsenate, but in 10^{-3} M. after 20 hours here is a sharp rise in the oxygen uptake and this is correlated with a rapid rowth of bacteria, presumably utilizing the material exuding from the dead r dying sections in that high concentration.

Thus there is no doubt that, in spite of the superficiality of his technique, onner's conclusions, that arsenate has a marked differentiating action on rowth and respiration, were correct. When 'penetration' effects are accounted or, growth inhibition becomes apparent at concentrations of the order of 0^{-5} M., whereas respiration begins to be affected by concentrations just above 0^{-3} M. and then only after 15–20 hours' exposure. It is conceivable that hese latter inhibitions are not due to a direct effect of arsenate on the respira-ory enzyme systems but to indirect effects arising from abnormal metabolism these moribund cells.

In addition to following arsenate effects on the respiration of sections in p.p.m. IAA alone, other experiments were carried out on sections growing a sucrose medium. It has already been seen that sucrose has no effect, etectable by the present techniques, on the arsenate inhibition of growth. n Fig. 7 is shown a typical drift curve of the respiration of sections in 2 per ent. sucrose. It will be seen that immediately after excision and placing ections in this sugar the respiration rises steeply in the first 2–3 hours and hence falls along a smooth curve running closely parallel to the normal rift in IAA alone. If the ratio of these two drifts in comparable samples is orked out a very constant value is obtained for the whole of the subsequent eriod up to 30 hours. With 2 per cent. sucrose this augmented respiration is a level about 70 per cent. above control. This confirms the view that, quite part from the availability of carbohydrate substrate, some internal factor is ausing a fall in respiration in these sections under both these conditions.

When the effect of arsenate is studied in the presence of sucrose we observe arked differences from the results in IAA alone (Fig. 8B). In the first place a eduction in the height of the initial sucrose stimulation is quite clear, even in 0^{-4} M. arsenate, and this inhibition becomes much more marked after 5–10 ours. It is as if the tipping in of arsenate immediately stopped the rise in espiration due to the sucrose. Later on, however, this inhibition is masked y an early and steeper rise due to bacterial growth, which is more prolific han in IAA alone and is discernible in concentrations of 10^{-4} M. arsenate. he cause of the greater sensitivity of the respiration in sucrose is entirely a atter for speculation. The fact that it can be detected almost immediately fter tipping suggests that the action is being exerted at the surface of the

sections and not at the respiration centres where the arsenate may not ye have penetrated. It is logical to deduce from this that the arsenate is prevent ing the entry of sucrose and thereby its subsequent stimulation of respiratio rate. Such a blocking action could be easily explained in terms of a recen theory of sugar absorption involving phosphorolytic breakdown of the sucros molecule at the cell surface (Dormer and Street, 1949). The arsenate woul presumably act by competitive inhibition of this phosphorylation.

DISCUSSION

The major point which is apparent from these few restricted investigation is the considerable danger inherent in a superficial and uncritical use of excise organ segments for the study of growth processes, particularly in relation t the effect of active chemical agents applied in the external medium. It has bee only too easy to measure simply the increase in bulk of a cell or organ and t identify this with the cell's 'growth'. Thus imperceptibly the essentiall dynamic nature of growth has been relegated to the background.

It must be remembered that an isolated growing segment of a plant orga is not a system of constant properties or potentialities; it is from the moment o its excision to the cessation of its growth a constantly changing system, no only from the point of view of its structure and its metabolism, but, what i more pertinent to the present paper, also in its *reactions* to any external en vironment. It is contended that the magnitude and time relationships of th growth-*rate* curve of such a system are of more fundamental importance an value than the simple measure of its integral at an arbitrary time—the so called 'total growth' of much recent work. This is particularly true when study is being made of the effects of any condition imposed on the growin cell and a careful investigation of time relationships of growth rate shoul constitute the basic approach to the problem. The principle behind this i well illustrated by the results of pea epicotyl growth in 10^{-5} M. arsenat Here there is an initial stimulation of the growth rate followed by an in hibition as growth proceeds, the total integrated effect in terms of 'tota growth' being scarcely significant. The discrepancy between results of Christi ansen and Thimann and of Galston and Hand on the effect of sucrose on pe epicotyl growth may well be resolved on reinvestigation with due care an proper attention to this time factor.

Turning specifically to inhibitor studies on isolated organ sections we ca visualize that three distinct time sequences are involved. Firstly there is th complex sequence of biochemical and structural changes in the cell itse which we term its growth. Secondly there is a sequence of changes in the ce directly associated with the applied inhibitor. The particular one which con cerns us is the inhibitor concentration change at the site of its action in the cel the so-called growth centre. Thirdly we have the possibility, which can in n wise be ignored, of changes in the *reactivity* of the cell-growth centres to th inhibitor itself. These last two time-sequences have been largely ignored i

growth inhibition studies with isolated organ segments, the 'total growth' being taken as the only measure of response. The resultant distortion of the concentration-inhibition relationships are well illustrated in the results from pea epicotyls and roots.

The fact that inhibitors require a definite period to attain their maximum activity at the growth centre and that this period may last, when low concentrations are used, for the major part of the grand period of growth of the cell, means that the effects of such inhibitor concentrations on the most important stages of growth, i.e. the stage of maximum growth *rate*, may be impossible of study with the isolated segment technique. These difficulties might be avoided, at least partially, by applying the low inhibiting concentrations *before* excision so that the equilibrium concentration of the inhibitor at the growth centre is reached as early as possible in the growth cycle of the excised tissue.

Turning specifically to the action of arsenate, the experiments reported suggest that we can distinguish a series of distinct stages. Firstly there is an immediate adsorption of the arsenate at some interface in the outside surface of the tissue. Here the phosphorolytic entry of sugar may be interfered with. From this adsorbed condition arsenate is released, whence it slowly migrates, at a rate proportional to its adsorbed concentration, to the centres of stretching growth (possibly within the cell wall). Here growth is progressively inhibited until an equilibrium concentration is reached or, with higher external concentrations, growth is completely stopped. Further migration of the arsenate takes it to the main respiratory centres where endogenous respiration may be inhibited. Whether the low sensitivity of respiration is real or apparent and due to this slow penetration to the respiration centres is a matter for future investigation.

Clearly the penetration factor has not been given enough attention in growth studies with excised organ sections involving active materials applied externally. Many of the interaction effects reported in the literature between chemical growth factors may quite possibly be explainable in terms of impeded or accelerated penetration and not, as is the present fashion, on a basis of competitive action at a specific enzyme system controlling growth. The determination of the precise shape of the growth curve should become a routine matter in all studies of this sort involving excised sections in which only the later stages of extension growth can be studied and in which most of the growth after excision may be over before the experimental treatment can attain its maximum effect.

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Studies on Plant Growth Hormones

I. BIOLOGICAL ACTIVITIES OF 3-INDOLYL-ACETALDEHYDE AND 3-INDOLYLACETONITRILE

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SUMMARY

1. Two neutral plant hormones, one isolated recently from plants (3-indolylacetonitrile) and the other (3-indolylacetaldehyde) reported to be present in plants, are available as pure synthetic compounds for investigation of their biological activities. This paper is mainly concerned with their effects on cell-elongation in the *Avena* coleoptile.

2. 3-Indolylacetaldehyde is considerably less active than 3-indolylacetic acid in the *Avena* straight-growth test; for example, a 1·0 mg./l. solution of the aldehyde shows an activity equivalent to that of a 0·1 mg./l. solution of the acid.

3. An acidic substance is produced in solutions of the aldehyde during the period of assay. In some experiments it accounts for all of the activity shown by the aldehyde solutions, on the assumption that it is 3-indolylacetic acid, and in other experiments it shows a greater activity than that of the aldehyde solutions from which it was obtained. Therefore, it is concluded that 3-indolylacetaldehyde itself is either inactive or inhibitory. Acid production in aldehyde solutions *in vitro* is much lower, a fact which suggests that there is enzymatic oxidation of aldehyde to acid in the presence of coleoptiles.

4. The activities of 3-indolylacetaldehyde in the pea test and in root-inhibition and of 3-indolylacetone in the straight-growth test are briefly reported.

5. 3-Indolylacetonitrile is considerably more active than 3-indolylacetic acid in the *Avena* straight-growth test; for example, a 0·1 mg./l. solution of the nitrile shows an activity equivalent to a 1·0 mg./l. solution of the acid. The inhibitory effect at concentrations above 1·0–10·0 mg./l. is less with the nitrile than with the acid.

6. There is negligible production of acid in solutions of the nitrile both *in vitro* and in the presence of *Avena* coleoptiles at temperatures ranging from −18° to 25° C. for varying lengths of time. The possibility of enzymatic conversion of nitrile to acid inside the cells of the coleoptile is discussed.

7. The activities of 3-indolylacetamide and of 2:4-dichlorophenoxyacetic acid and the corresponding nitrile are considered in this connexion.

8. The nitrile is destroyed by treatment with alkali but not by acid. In the light of these results, it is desirable to re-examine previous work on identification of auxins in plants by their acid and alkali sensitivity. Evidence for the existence of the nitrile in a number of other plants is presented.

INTRODUCTION

THERE have recently been several reports on the existence in plant tissues f a neutral hormone. It has been suggested, on the basis of chemical tests nd by enzymatic oxidation of this compound to an acidic auxin, that it is

3-indolylacetaldehyde (work in this field is summarized by Larsen, 1951*a*). However 3-indolylacetaldehyde (IAc) has not been isolated in a pure form from plant tissues, so that evidence for its existence in plants remains indirect. Moreover, work on its biological properties has so far been tentative because synthetic aldehyde has not been available in the chemically pure state. From work on crude 3-indolylacetaldehyde it is uncertain whether this compound functions as a plant-growth hormone and it has been suggested that activity in the *Avena* curvature test is due to the production of 3-indolylacetic acid (IAA) from the aldehyde.

The aldehyde has now been synthesized and isolated in a pure form (Brown *et al.*, 1952*a*) and it is therefore possible to construct standard activity curves relating biological effect to actual concentration. A preliminary account of the work on indolylacetaldehyde reported here is given by us in the above publication.

Parallel with the work on 3-indolylacetaldehyde, a detailed examination of the neutral hormone of Brussels sprouts and cabbage has been carried out jointly by the Departments of Botany and Chemistry of Manchester University. One of us (J. A. B.), in an investigation on methods of extraction of plant hormones, found that ether extracts of fresh Brussels sprouts at 8° showed high activity in the *Avena* straight-growth test. There are earlier reports on the high auxin content of members of the Cruciferae (Linser, 1940; Avery *et al.*, 1945), and Larsen (1944) demonstrated the existence of a neutral hormone in cabbage. Recently Linser (1951) has reported that cabbage contains at least two growth hormones, which are easily separable by chromatographic methods.

An investigation into the nature of the highly active auxin in cabbage, undertaken by the Chemistry Department, led to the isolation of a pure crystalline neutral hormone which has been identified as 3-indolylacetonitrile (IAN) (Jones *et al.*, 1952). Synthetic nitrile is available, and it is therefore possible to construct standard activity curves for this substance also.

Thus, two neutral substances, one known to exist in plant tissues (IAN) and the other (IAc) reported to be present on the basis of indirect tests, are available for detailed biological study.

The present paper is concerned mainly with the effect of these substances on cell-elongation in isolated *Avena* coleoptile sections, and on production of acid in solutions of the two compounds *in vitro* and in the presence of coleoptile sections. The significance of the results from these and from chemical experiments with the nitrile is discussed.

EXPERIMENTAL TECHNIQUES

The assay technique used in the present investigation has already been described (Bentley, 1950). Briefly, unhusked oats are grown in a coarse sand at 25° and 90 per cent. relative humidity until the coleoptiles are approximately 1·5–2·0 cm. long. Coleoptiles of uniform length (usually 1·4–1·6 cm.) are selected, 10-mm. sections cut from them, the sections mounted on glass

capillaries and floated on the solution to be tested. Elongation of the sections after 20 hours is measured. The standard technique is to have 10 sections in 10 ml. of solution in a small Petri dish, 5 cm. diameter. In the present experiments, usually 30 or 50 replicates were tested at each concentration (i.e. 3 or 5 dishes) except in experiments assaying the concentration of acid in solutions of the aldehyde or nitrile, when usually 10 replicates were tested at each concentration. Variation is indicated in the figures by vertical lines on the graphs showing twice the standard error at each point.

Production of acidic growth-promoting material in solutions of the two substances in the presence of *Avena* coleoptile sections and *in vitro* was determined as follows. Solutions were assayed in the usual manner, using 5 dishes (i.e. a total volume of 50 ml.) at the 10 mg./l. concentration. Control solutions were kept under identical conditions but without coleoptile sections. The acid and neutral fractions of both sets of solutions were then separated, and the activity of each determined by subsequent bio-assay. To separate the acid and neutral fractions, solutions were made alkaline with 1 per cent. sodium bicarbonate, shaken three times with ether to remove the neutral fraction; the bicarbonate solution was then acidified with 5 per cent. sulphuric acid to approximately pH 3·0 and shaken again three times with ether to remove the regenerated acid fraction. Ethereal solutions of acid and neutral fractions were washed twice with distilled water, evaporated to approximately 5 ml., water added to the same volume as the original aqueous solution (except where otherwise stated) and the remainder of the ether removed under reduced pressure at a water-bath temperature of 60°–80°.

3-INDOLYLACETALDEHYDE

The stability of 3-indolylacetaldehyde was not known and therefore care was taken to ensure that the minimum of chemical change occurred in solutions for assay. Aqueous solutions were prepared immediately before use from ethereal solutions of the aldehyde (kindly supplied by the Chemistry Department) which had been stored for not longer than a week at a temperature of −18°.

A typical experiment on the activity of IAc compared with that of IAA is shown in Fig. 1. The solution of aldehyde used in this experiment was tested before assay for the production of acid by splitting a portion into acid and neutral fractions and assaying. The acid fraction was inactive, showing that there is no acid in a freshly prepared solution of the aldehyde. Results of this and similar experiments show that a 1·0 mg./l. solution of IAc has an activity equivalent to a 0·1 mg./l. solution of IAA. The activity falls at higher concentrations, so that 10 mg./l. solutions of IAc have an activity equivalent to a concentration of approximately 0·2 mg./l. IAA. The activity rises at lower concentrations, a 0·1 mg./l. solution of the aldehyde having an activity equivalent to a 0·03 mg./l. concentration of acid.

To determine the production of acid in the presence of coleoptiles during the assay period (20 hours), the staled 10 mg./l. solutions of aldehyde from the

experiment shown in Fig. 1 were separated into acid and neutral components which were assayed separately at successive dilutions. The results show (Fig. 2) that the activity of the neutral fraction is of the same order as that of the

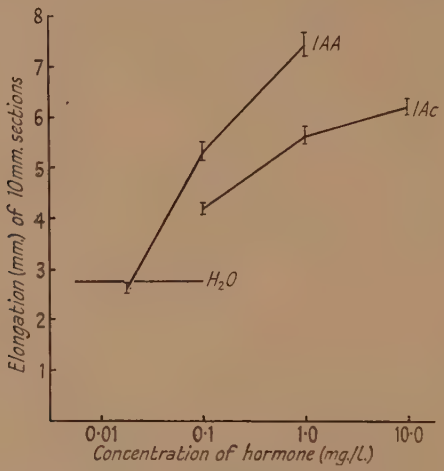

FIG. 1. Relative activities of IAA and IAc in the *Avena* straight-growth test.

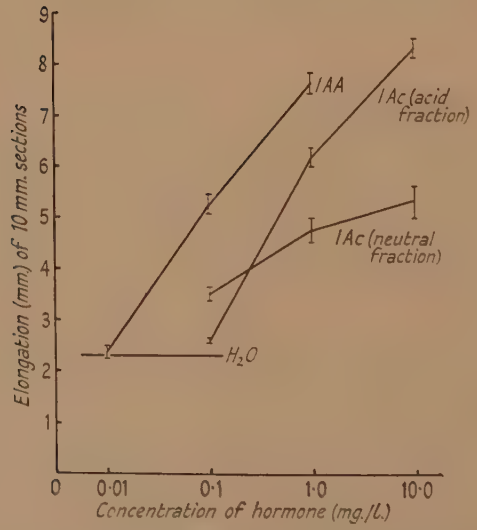

FIG. 2. Production of acid in solutions of IAc in the presence of coleoptile sections.

original solution, though slightly lower. There is, however, pronounced activity in the acid fraction. The acid solution at the first dilution, i.e. equivalent to a concentration of 1 mg./l. of the aldehyde, has an activity correspond-

ing to a 0·2 mg./l. concentration of IAA, and the activity curve is similar. In another experiment the acid fraction equivalent to a 1 mg./l. solution of the aldehyde had an activity corresponding to a 0·1 mg./l. concentration of IAA. Thus the activity of the acid growth-promoting substance produced during the period of assay is as great or greater than that of the aldehyde solution from which it is produced.

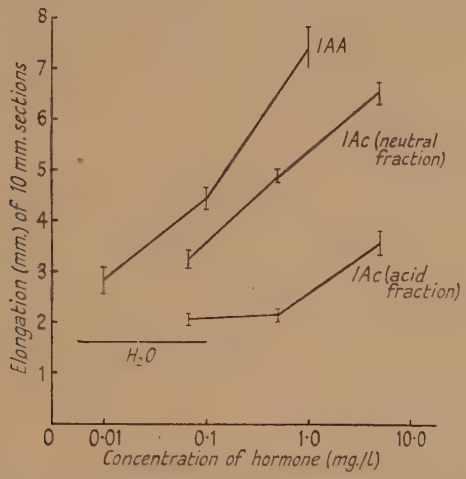

FIG. 3. Production of acid in solutions of IAc without coleoptile sections.

Control solutions of IAc, placed under identical conditions (25° for 20 hours) but without coleoptile sections, were also separated into acid and neutral fractions, which were assayed to determine the amount of acid produced *in vitro*. The results show (Fig. 3) that only approximately 1 per cent. of the activity is caused by acid, since a 5 mg./l. solution of the acid fraction has an activity approximately the same as that of a 0·05 mg./l. solution of the neutral fraction. (In this experiment the acid and neutral fractions were made up to twice the volume of the original solutions; therefore the concentrations tested were only half those shown in other figures.) Thus, *in vitro* change does not account for all the production of acidic material in the presence of coleoptiles.

DISCUSSION ON 3-INDOLYLACETALDEHYDE

Larsen (1944, 1949) found that impure preparations of IAc caused lower maximum angles in the *Avena* curvature test than IAA. Two explanations have been offered for this. Skoog (1947) suggests that IAc is inhibitory in the curvature test; Larsen (1949) thinks that its movement might be non-polar in the coleoptile and that, by spreading across to the side opposite the agar block, it might cause a growth effect which would reduce the curvature.

Results on the activity of pure IAc in the *Avena* straight-growth test, reported in this paper, show that the maximum activity obtained is lower than with IAA in this test also. Larsen's explanation for low activity is not possible in the straight-growth test, which does not depend on a differential growth-response.

Results also show that an acidic substance is produced in solutions of aldehyde in the presence of coleoptiles. This substance, which is probably IAA, accounts in some experiments for all of the activity of the aldehyde solution from which it was produced, and in other experiments shows a greater activity than that of the aldehyde solution. There is thus no evidence for any growth-promoting activity of IAc, and it can be concluded either that IAc is inactive or that it is an inhibitor.

Production of acid from aldehyde solutions *in vitro* is much lower, a fact which suggests that there may be enzymatic oxidation of aldehyde to acid in the presence of coleoptiles. No attempt has been made to investigate this aspect of the problem. Larsen (1949) put forward strong evidence for the enzymatic conversion of aldehyde to acid in impure preparations. In this connexion it is noteworthy that 3-indolylacetone (synthesized by Brown *et al.*, 1952*b*), a compound closely related to IAc, is virtually inactive in the straight-growth test; from the chemical structure it is unlikely that this compound would oxidize easily to the acid.

No attempt has been made in the present work to determine accurately the amount of acid produced per mole of aldehyde destroyed in the presence of coleoptiles, as Larsen did for indoleacetaldehyde (1949) and naphthaleneacetaldehyde (1951*b*). Only limited amounts of material were available and more experiments are needed before accurate calculations can be made. Moreover, no account has been taken in the above experiments of the destruction of acid during bio-assay. Preliminary experiments on the destruction of IAA in the straight-growth test have shown that 30–50 per cent. IAA is used or inactivated in 1 mg./l. solutions and 60–80 per cent. in 0·1 mg./l. solutions. This factor needs to be taken into account when considering production of acid during bio-assay.

Preliminary observations have also been made on the activity of IAc in root inhibition and in the pea test. The studies on root inhibition were carried out by the senior author using a modified form of Moewus's cress test (Moewus, 1949) in which the substance to be examined is incorporated in 1·5 per cent. agar solution on which the roots are grown instead of on filter-paper. This method is discussed in greater detail in the second paper of this series. Results show that IAc is approximately 5 to 10 times less active than IAA in both tests over a range of concentrations from 0·01–10·0 mg./l. It would be useful to determine the production of acid in solutions of IAc in the presence of pea stems and cress roots in the same way as with coleoptile sections. There is evidence (already discussed) for the existence in coleoptiles of an enzyme system which converts IAc to IAA, but it cannot be assumed that similar systems occur in other plant tissues.

3-INDOLYLACETONITRILE

As mentioned in the introduction, several workers have reported the presence of a neutral hormone in plants, suggested by some to be 3-indolylacetaldehyde. It is now established that the neutral hormone of cabbage is 3-indolylacetonitrile (conveniently referred to as IAN) by isolation and characterization of the pure crystalline material (Jones *et al.*, *loc. cit.*). During the course of the isolation biological activity was followed by the *Avena*

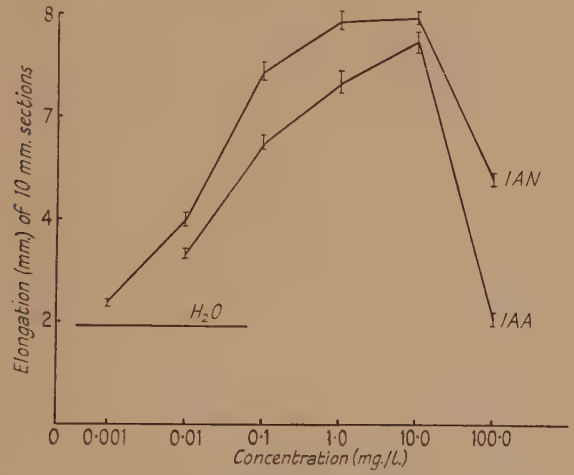

FIG. 4. Relative activities of IAA and IAN in the *Avena* straight-growth test.

straight-growth method, and it became evident that the neutral hormone was more active than IAA. Thus, in many assays, solutions of the neutral hormone of approximately 0·1 mg./l. had activities equal to 0·5–1·0 mg./l. solutions of IAA. More concentrated solutions of the neutral hormone could not be expressed in terms of IAA, as they showed activities greater than the maximum obtained with IAA. These results are in sharp contrast to those obtained with 3-indolylacetaldehyde.

When the neutral hormone was identified, synthetic IAN was prepared, and it was possible to construct accurate activity curves of biological response against concentration. Results of a typical experiment in the *Avena* straight-growth test show (Fig. 4) that IAN is more active than IAA in promoting cell-elongation over a range of concentrations from 0·001–100 mg./l.; a 0·1 mg./l. solution of the nitrile has approximately the same activity as a 1·0 mg./l. solution of the acid (i.e. at this concentration IAN is approximately 10 times as active as IAA). The inhibitory effect at concentrations above 10 mg./l. is less with the nitrile than with the acid. In other experiments at 1,000 mg./l., both the nitrile and the acid cause complete inhibition of growth; it has been noticed that sections in IAN solutions shrink slightly (about 0·5 mm.), although this is not obtained with sections in IAA solutions.

Results of a number of further experiments comparing the activities of IAA and IAN are shown in the table, and illustrate the amount of variation existing from occasion to occasion. Standard errors are not given, as variation among replicates in the straight-growth test is adequately illustrated in Figs. 1–4. Figures for response in IAA and IAN in the first 8 experiments of the table are means of 30 replicates, in the remaining 7 experiments of 10 replicates. Figures for water are the means of 10 replicates throughout. Oats used for experiments B. 147–B. 170 were harvested in 1949 and those used in experiments B. 171–B. 175 in 1950.

TABLE I

Elongation (mm.) of 10-mm. sections in solutions of IAA, IAN and water in the Avena *straight-growth test*

Expt. No.	Concentration of hormone (mg./l.) IAA							IAN							Water
	1,000	500	100	10	1·0	0·1	0·01	1,000	500	100	10	1·0	0·1	0·01	0·001	
B. 151	—	—	—	—	6·8	4·0	2·2	—	—	4·4	6·7	7·1	5·7	2·5	2·0	2·0
B. 155	—	—	—	—	6·4	5·0	3·3	—	—	5·7	7·9	7·8	6·6	4·1	3·2	3·0
B. 157	—	—	2·6	8·0	7·9	6·0	3·1	—	—	4·8	7·3	7·6	5·6	3·6	2·0	1·9
B. 160	—	—	2·1	7·5	6·7	5·5	3·3	—	—	4·8	8·0	7·9	6·9	4·0	2·4	1·9
B. 162	—	—	2·4	8·2	8·3	5·9	4·3	—	—	5·0	7·6	6·8	5·9	4·3	2·9	2·7
B. 163	—	—	2·6	8·4	7·8	5·7	3·4	—	—	5·1	8·5	8·1	7·4	4·6	3·0	2·1
B. 164	—	—	2·3	7·2	6·6	4·5	2·7	—	—	4·1	7·5	8·3	7·7	4·1	2·4	2·1
B. 165	—	—	2·6	8·3	8·0	6·1	3·2	—	—	4·7	7·8	8·0	6·9	4·0	2·3	3·0
B. 147	—	—	—	—	6·4	4·7	2·4	—	—	4·8	8·0	7·8	6·6	3·4	2·7	1·9
B. 169	—	—	2·5	7·9	7·6	5·4	2·5	—	—	4·0	7·5	7·5	7·2	4·3	2·3	1·9
B. 170	—	—	0·9	5·6	6·0	5·2	3·7	—	—	4·8	7·4	7·0	6·8	4·3	2·3	1·7
B. 171	0·55	—	5·8	7·9	7·6	5·5	2·6	−0·3	2·0	7·4	7·9	8·2	7·1	5·0	3·1	3·2
B. 172	0·5	1·5	5·8	7·6	6·8	4·5	2·5	−0·6	1·6	6·3	6·3	7·7	6·2	4·2	3·9	2·7
B. 173	0·1	0·8	6·3	7·5	6·3	4·4	1·7	−0·8	1·5	7·4	7·9	8·3	6·5	4·3	3·2	1·8
B. 175	−0·3	—	1·4	7·5	6·4	4·9	2·7	−0·3	—	6·3	7·1	7·3	6·2	3·6	1·9	1·7

It is well known that the response of coleoptiles to hormone solutions varies from occasion to occasion, and it has been suggested (Bentley, 1950) that this is due to germination conditions causing variation in factors such as rate of imbibition of water and mobilization of enzymes. Such factors must ultimately determine the type of coleoptile produced. Results in the table show that variation in response to IAN on different occasions differs from variation in response to IAA. Thus, although the majority of experiments show that IAN is approximately 10 times more active than IAA at a concentration of 0·1 mg./l., activities considerably greater than 10 times that of IAA (B. 164, B. 170) or lower than that of IAA (B. 157, B. 162) are sometimes obtained.

The variation in relative activities of the two substances made it difficult to calculate the amounts of neutral hormone present at different stages during the initial isolation of the nitrile, and it is suggested that it might be advisable, in future isolations where the presence of the nitrile is suspected, to use synthetic IAN as a control rather than IAA. The fact that response to the nitrile does not always vary to the same degree as response to IAA does not necessarily mean that the two substances act through different mechanisms, since differences in secondary factors such as permeation through cell-membranes might be involved.

Studies on the production of acid in solutions of IAN *in vitro* and in the presence of coleoptile sections have been carried out using the same technique as in the indolylacetaldehyde studies. In 10 mg./l. and 100 mg./l. solutions of IAN kept at temperatures varying from room temperature to −18° for periods up to 63 days, production of acid was never higher than an amount equal to a concentration of IAA of 0·3 per cent. of the original concentration of nitrile, and in most of the experiments it was negligible. Similarly, a negligible amount of acid was produced in 10 mg./l. solutions of IAN *in vitro* and in the presence of coleoptile sections at 25° for 20 hours. These results show that solutions of the nitrile are very stable, even in the presence of coleoptile sections, in marked contrast with solutions of IAc.

DISCUSSION ON 3-INDOLYLACETONITRILE

Results of *Avena* straight-growth tests show that IAN is more active than IAA in promoting cell-elongation over a range of concentrations from 0·001 to 100 mg./l. (For strict comparison results should be expressed in terms of molarity rather than as actual concentrations, but this would cause very little difference in the relative positions of the curves.) Obviously it is necessary to consider this high activity further, since it has hitherto been generally accepted that only molecules with a side-chain containing a carboxyl group have auxin activity.

Some nitriles have been tested previously for biological activity, and it has been suggested that they owe their activity to hydrolysis to the corresponding acids (Veldstra, 1944; Thimann, 1951). From results reported in this paper, however, there is little evidence for the production of acid in solutions in which coleoptile sections are immersed. This means that, if hydrolysis of nitrile to acid does occur, then it is confined within the cells of the coleoptile, and the product cannot be detected in the external medium, as it can in the oxidation of IAc to IAA by coleoptile tissues. If acid is formed within the tissues in both reactions it is difficult to see why it should be easily liberated to the external medium in one reaction and not in the other. It is more probable that the mechanism responsible for oxidation of the aldehyde can act on aldehyde molecules in the external medium (for example, if it is an enzymic oxidation, the enzymes may be situated in the surface layers of the coleoptile tissues, or may diffuse into the external medium), whereas hydrolysis of the nitrile, if it occurs, is localized within the cells. Obviously it is necessary to investigate further the question of the production of acid in solutions of the aldehyde and the nitrile in the presence of coleoptile tissues.

The fact that the nitrile shows higher growth-promoting activity than the acid is difficult to explain if the nitrile only possesses activity by reason of its conversion to the acid. Evidence presented in Part II of this series shows, however, that IAN may be transported in coleoptile tissues more readily than IAA. From a consideration of these data it is possible that, if conversion of nitrile to acid does occur in the plant tissues, then higher concentrations of acid might be produced from the nitrile within the cells than could be achieved

by penetration of the acid itself. This could account for the greater activity of the nitrile in promoting cell-elongation, but not for the fact that the nitrile inhibits less than the acid at high concentrations.

Hydrolysis of the nitrile to the acid is equivalent to the production of the ammonium salt of the acid. It is therefore necessary to consider the activity of the latter, and experiments at present in progress show that equimolecular

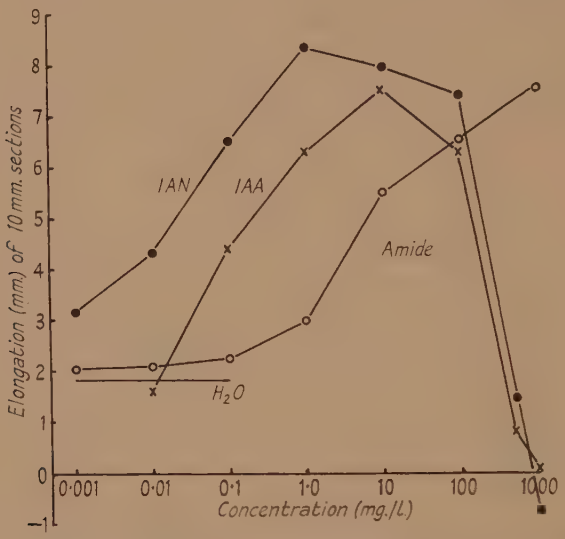

FIG. 5. Relative activities of IAA, IAN, and 3-indolylacetamide in the *Avena* straight-growth test.

solutions of the ammonium salt of IAA, and mixtures of IAA and ammonia, do cause higher activity at certain concentrations in the straight-growth test than IAA alone. These experiments will be reported in full later.

It may be noted in this connexion that 3-indolylacetamide, which is obviously a possible intermediate in the conversion of nitrile to acid, shows an activity which can be explained entirely on the assumption that IAA is present in the solutions in concentrations equal to 1–3 per cent. of the concentrations of the amide (Fig. 5). This small amount of acid might have been present in the original preparations of the amide.

IAA causes greater inhibition than IAN at concentrations above 10 mg./l. If there is enzymatic conversion of nitrile to acid, this fact could be explained by assuming that conversion is inhibited at higher concentrations. Alternatively, inhibition may be caused by secondary properties of active molecules, and these properties may come into operation in solutions of IAA at concentrations below the optimum for auxin action.

A different relationship exists between the activity curves of 2:4-dichlorophenoxyacetic acid and the corresponding nitrile in the straight-growth test (Fig. 6). 2:4-dichlorophenoxyacetonitrile has higher growth-promoting acti-

vity than the acid at low, and greater inhibitory activity at high, concentrations. If there is conversion of the nitrile to the acid in the tissues of the coleoptile, such results can be explained entirely by assuming greater ease of entry of the nitrile. The nitrile curve is similar in shape to the acid curve, but is displaced to lower concentrations relative to the acid curve. On this hypothesis there is no need to suppose different inhibitory mechanisms for these two substances

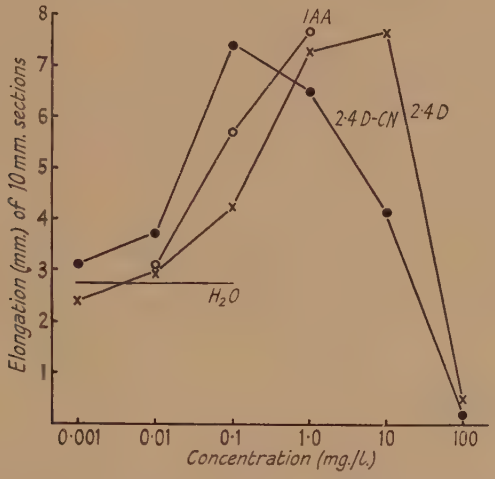

FIG. 6. Relative activities of IAA, 2:4-dichlorophenoxyacetic acid (2·4 D) and 2:4-dichlorophenoxyacetonitrile (2·4 D–CN) in the *Avena* straight-growth test.

at high concentrations. The activity curves for 1-naphthylacetic acid and the corresponding nitrile show a similar relationship.

It was thought in the early stages of investigation, before the neutral hormone was identified, that it might act synergistically with the native hormone of the coleoptile, causing apparent high activity. This point was investigated briefly by the senior author by comparing the activities of IAA and IAN (from concentrates of the naturally occurring hormone) in the *Avena* straight-growth test on three types of coleoptile sections, viz. normal coleoptile sections, sections from coleoptiles which had been decapitated 2 hours before use and therefore contained no free auxin, and sections from deseeded coleoptiles (technique of Skoog, 1937) containing no free auxin and no 'auxin precursors'. IAN showed greater activities than IAA at comparable concentrations with all three types of coleoptile. Thus there is no evidence for a synergistic action between IAN and free auxin or 'auxin precursors' in the coleoptile. Furthermore, mixtures of IAN (the natural product) and IAA were tested in different proportions in the straight-growth test, and gave no evidence of a synergistic action. The activities of such mixtures can be fully explained in terms of the individual components. These experiments will be repeated with synthetic nitrile.

The high activity of IAN in the *Avena* straight-growth test can be used a an indication of its presence in plant extracts. For example, ether extracts of various members of the Cruciferae family (radish leaves; cauliflowe leaves, flowers, and stems; swede leaves, roots, and stems; turnip leaves roots, and stems) all contain neutral material which causes greater elongatio than the maximum obtained with IAA. As shown earlier, the activity of solu tions of 3-indolylacetaldehyde (IAc) is considerably lower than that of IAA Therefore the neutral hormone in such extracts is more probably the nitrile Also, it was mentioned earlier that the presence of a neutral hormone in number of other plant tissues has been reported by several workers, som of whom suggested that IAc is the active compound (Larsen, 1951*a*, *loc. cit.*) In the light of the present work it is clearly desirable to re-examine the natur of the neutral hormone in these plants.

Chemical properties of the nitrile such as acid and alkali stability have als been examined, and will be reported in full in the chemical literature. We ar indebted to Dr. G. Smith for the following brief report. The nitrile is de stroyed by alkaline hydrolysis, being hydrolysed to acid by treatment with N.NaOH at 100° for 30 minutes, but not by treatment with acid (1 N.H_2SO at 100° for 1 hour). Thus the behaviour of the nitrile is parallel with tha reported for auxin *a*, except that another active acidic substance (IAA) i produced by alkaline hydrolysis if the alkaline fraction is re-acidified before extracting with ether. The active Na and K salts of IAA, present in alkalin fractions, would not be extracted by ether. It is therefore necessary to re investigate the nature of the hormones in plants in which auxin *a* is reporte to be present on the basis of acid and alkali stability tests, and in materia in which IAA is reported to be present after treatment with alkali (examples o both types are summarized by Söding, 1952, pp. 74 and 78).

Further biological properties of the nitrile are reported in the next pape in this series.

The authors wish to express their thanks to the Agricultural Researc Council for grants which have made this work possible, to Professor S. C Harland for generous facilities in the Department of Botany, to Professo E. R. H. Jones and other members of the Chemistry Department for thei close co-operation throughout the work, to Mrs. V. Shaw for technica assistance with the assays, and to Dr. W. A. Sexton, Imperial Chemica Industries Ltd. (Dyestuffs Division) for kindly supplying the 2:4-dichloro phenoxyacetonitrile.

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[1] These extracts were prepared by Dr. S. Dunstan of the Chemistry Department. Frozen crushed material was extracted with peroxide-free ether for 20 hours at −7°, and the extract were separated into acid and neutral fractions.

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NOTE

Osborne (*Nature*, **170**, 210) has recently suggested, on the basis of experiments showing a synergistic action between IAA and IAN in the pea test (also obtained in his laboratory) that a similar effect is responsible for the high activity of IAN in the *Avena* straight-growth test. This assumes that hydrolysis of IAN to IAA occurs in the presence of *Avena* tissues. Experiments reported on p. 401 of this paper show that there is no evidence for this assumption, and experiments reported on p. 403 show that there is no evidence for a synergistic action in *Avena* between IAN (natural product) and applied IAA, or between IAN and the native auxin of *Avena* coleoptiles. This question will be discussed further in a later paper.

Studies on Plant Growth Hormones

II. FURTHER BIOLOGICAL PROPERTIES OF 3-INDOLYLACETONITRILE

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SUMMARY

1. 3-Indolylacetonitrile is more active than 3-indolylacetic acid in the *Avena* straight-growth test, but less active in the *Avena* curvature test at comparable concentrations. Reasons for this are discussed, and results of previous work on plant extracts using the curvature test as a means of assay are considered.

2. Transport of both the acid and the nitrile is polar, from apex to base of the coleoptile. The nitrile can reach the growing cells as easily, and possibly more easily, than the acid. The significance of these findings for a theory on the mechanism of action of the nitrile is discussed.

3. The nitrile is inactive in the pea curvature test and straight-growth of pea stem sections except at high concentrations. It is also inactive or only slightly active in lateral bud inhibition, root initiation, and petiole abscission at the concentrations tested.

4. It is less active than the acid in root inhibition in cress, but approximately as active in *Avena*. It is approximately as active as the acid in parthenocarpic fruit development, and initiation of cambial activity.

5. The significance of these results is discussed.

1. INTRODUCTION

A NEUTRAL hormone, highly active in causing cell-elongation in the *Avena* coleoptile, has been isolated from cabbage and identified as 3-indolylacetonitrile (Jones *et al.*, 1952). Its effect in the *Avena* straight-growth test is reported in detail in the first paper of this series (Bentley and Housley, 1952), in which it is shown that the nitrile is considerably more active than the corresponding acid, 3-indolylacetic acid (IAA). In an attempt to investigate this high activity, experiments on the *Avena* curvature test and on transport through coleoptile tissues have been carried out, and are reported in this paper.

In addition, it is now well established that hormones affect many other aspects of plant development as well as cell-elongation in *Avena*. For example lateral bud inhibition, cambial initiation, parthenocarpic fruit development root initiation, root growth, and various other aspects of plant growth have been investigated (summarized by Bonner, 1950, chap. 29). These studies have largely been carried out using IAA, because this was, until recently, the only hormone known to exist in plant tissues. It is now necessary to carry out similar investigations with 3-indolylacetonitrile (conveniently referred to as IAN), and this paper presents preliminary results on the effect of IAN on

number of aspects of plant development. The results of the investigation have already been reported in brief (Jones *et al.*, *loc. cit.*).

2. *AVENA* CURVATURE TESTS

It has been shown in the first paper in this series that IAN is more active than IAA in the *Avena* straight-growth test. Thus, in typical experiments a 0·1 mg./l. solution of the nitrile has approximately the same activity as a

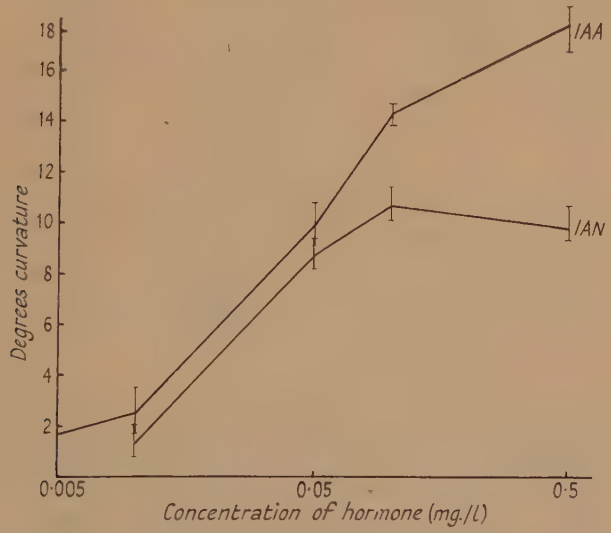

FIG. 1. Effect of IAA and IAN in the coleoptile curvature test. Each point is the mean of 20–30 replicates. Vertical lines denote 2 × standard error at each point.

1·0 mg./l. solution of the acid. Also, the inhibitory effect at concentrations above 1·0–10·0 mg./l. is less with the nitrile than with the acid. These results are not obtained in the *Avena* curvature test (technique of Rawes and Hatcher, 1948). In this test, IAN has approximately the same activity as IAA at concentrations of 0·1 mg./l. and lower (Figs. 1 and 2). At concentrations above 0·1 mg./l. the activity of IAN falls markedly below that of IAA and may disappear altogether while IAA is still causing definite curvatures. There is thus a marked difference between results obtained in the two tests. The significance of this finding is discussed in the light of the experimental results of Section 3.

It will be noted that the hormone concentrations in the figures have been plotted on a logarithmic scale, although previous workers have generally used an arithmetic scale for the curvature test. It has been found in this laboratory that the response obtained with IAA over a wide range of concentrations shows a linear proportionality to the logarithm of the concentration rather than to the concentration itself. The same relationship holds for coleoptile section growth and for root inhibition.

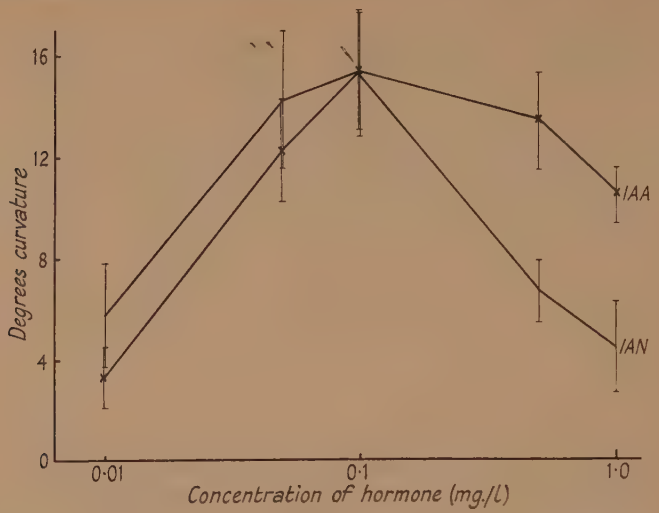

FIG. 2. Effect of IAA and IAN in the coleoptile curvature test. Each point is the mean of 20–30 replicates. Vertical lines denote 2 × standard error at each point.

3. TRANSPORT EXPERIMENTS

The contrast between the effects of the two substances in the straight-growth test and in the curvature test, both of which measure cell-elongation in coleoptiles, suggests that differences in permeation through cell-membranes or in transport to the growing zone might be involved. A preliminary examination of transport of IAA and IAN in the coleoptile was therefore undertaken. In these experiments 10-mm. coleoptile sections were supported vertically, either the right way up or inverted, on small glass capillaries embedded in wax. The lower end of the sections, with respect to gravity, rested on moistened filter-paper. Agar blocks containing the substance being tested were placed symmetrically on the upper ends. The sections were then allowed to stand in approximately 80 per cent. relative humidity at 25° for the period of the experiment, and the final length measured. Increase in length was taken as a measure of the ease with which the substance was transported, either up or down the coleoptile. The results show (Table I) that there is no growth greater than residual growth due to water uptake, when agar blocks containing either IAA or IAN at concentrations of 0·5–1·0 mg./l. are placed on the morphologically lower ends of coleoptile sections. Both substances, however, cause pronounced growth when applied to the morphologically upper ends. Therefore, transport of both IAA and IAN in the coleoptile (i.e. transmission of their effect through the growing cells) is strictly polar, from apex to base only.

It will be noted that some growth occurs in inverted coleoptiles when the concentration is high (Table I, Expts. 43 and 59); this is most probably due to diffusion of hormone down the outer surfaces of the coleoptile in water

films. Von Guttenberg's report (1950) that IAA moves from base to apex in the coleoptile may be the result of similar diffusion.

TABLE I

The effect of IAA, IAN and water applied to the upper ends of inverted and non-inverted coleoptile sections. Each figure is the mean of 10–15 replicates. NP denotes natural product

Expt. No.	Time (hrs.)	Test solution (mg./l.)	% elongation of sections	
			Inverted	Non-inverted
1	3	IAA 1·0	6	21
		IAN 1·0 (NP)	6	14
2	3	IAA 1·0	9	30
		IAN 1·0 (NP)	8	24
43	4	IAA 0·5	6	23
		IAN 5·0 (NP)	14	29
44	5	IAA 0·05	5	—
		Water	6	—
59	5	IAA 5·0	14	31
		IAA 0·5	9	28
		IAN 5·0	22	36
		IAN 0·5	10	29
		Water	9	16

Reversal of the normal effect of gravity on the tissues by inversion of the sections sometimes causes a slight reduction in residual growth due to water uptake (Table I, Expt. 59), suggesting that the lack of response to IAA and IAN of inverted coleoptile sections may also be due in part to alteration in gravitational effect rather than a property of the tissues. This possibility was examined in further experiments by applying the agar blocks to the upper or lower surfaces of non-inverted coleoptile sections. Under these conditions growth occurred only when the blocks were applied to the upper surfaces and not when they were applied to the lower surfaces. The results were of the same order as those given in Table I. Therefore, growth in coleoptile sections is clearly controlled predominantly by the polarity of the tissues and not by the effects of gravity on the tissues.

Further, it should be noted that the strictly polar basipetal transport of a neutral, non-ionized molecule such as the nitrile cannot be explained in terms of electrical polarity, as has been attempted for the acid.

An attempt was made to compare basipetal transport (or transmission of the effects) of IAA and IAN by applying the substances in agar blocks to the upper surfaces of non-inverted coleoptile sections, and comparing growth-responses. The usual method of measuring transport is to determine the amount of auxin released from the lower ends of such sections. The sections used in these studies are actively growing under the influence of auxin applied to the top surface. If the amount of auxin collected from the bottom surface is used as a measure of the amount transported from the top surface, then one is assuming either that the auxin travelling through the tissues is not consumed in the growth it causes in its passage down the coleoptile, or that one

molecule of auxin is released from the base of a growing cell for every molecule used in growth within the cell. Neither assumption appears justified on existing experimental evidence. It was considered that the amount of growth caused by the substance (i.e. increase in length of the section) was at least as good, if not a better, indication of the ability of the substance to get to the elongating cells. Using this criterion, results show (Table II) that usually there is greater growth with IAN than with IAA (seen in all the experiments except Nos. 56 and 57 A).

TABLE II

A comparison of elongation of coleoptile sections when IAA or IAN is applied to the morphologically upper surface

Expt. No.	Time (hrs.)	Concentration of hormone (mg./l.)	% elongation of sections IAA	IAN
53	24	0·5	30	34*
54	8	,,	23	26
56	4	,,	28	24*
57 A	24	,,	39	36
57 B	,,	,,	20	26**
59	5	,,	28	29
,,	,,	5·0	31	36

** = significant difference from the corresponding value for IAA at the 1 per cent. probability level, * = at the 5 per cent. probability level. Each figure is the mean of 10–15 replicates.

The agar blocks were replaced every 2 hours in Expt. 54 and once after 6 hours in Expt. 57 A; in the other experiments they were not replaced. The effect is small and is statistically significant in only two experiments (Nos. 53 and 57 B). In Expt. 56 IAA causes significantly more growth than IAN. A concentration of hormone of 0·05 mg./l. was also tried, but caused no greater growth than water. These experiments are preliminary and studies on transport of IAA and IAN in coleoptile tissues will be continued. Assuming, however, that the amount of growth is a fair measure of the amount of substance reaching the elongating cells, the experiments do show that IAN is transported basipetally through living coleoptile tissues as easily, and possibly more easily, than IAA.

The lower activity of the nitrile in the curvature test at concentrations above 0·1 mg./l. (Section 2) may therefore be due to one of two causes. The nitrile may get into the cells on the side of the coleoptile below the block more easily than the acid, and an inhibiting concentration may therefore be reached sooner than with the acid. In considering this possibility it must be remembered that the nitrile only inhibits growth in the cells of isolated coleoptile sections at concentrations considerably higher than those causing inhibition with the acid (Bentley and Housley, 1952, Fig. 4). A more likely explanation is that the nitrile spreads across the coleoptile when applied unilaterally in the curvature test, so that growth occurs on both sides, causing a reduction in the resultant curvature. This test is not therefore suitable as a means of assay of the nitrile.

If IAN is transported through tissues more easily than IAA this is the first auxin to show this property, as far as the authors are aware. The synthetic auxins so far examined are transported through coleoptile tissues less easily than IAA (Pincus and Thimann, 1948, p. 27). It may also be noted here that 2:3:6-trichlorobenzoic acid and the corresponding aldehyde, which both show high activity in the *Avena* straight-growth test (Bentley, 1950), are inactive in the curvature test, and also when tested by the same techniques as used in the present investigation, indicating that they are not transported through coleoptile tissues. Such observations confirm the suggestion that tests depending upon transport of the auxin to the growing region, as for example, in the curvature test, are not suitable as methods of assay for primary growth-promoting activity.

It has been suggested that nitriles owe their activity to hydrolysis to the corresponding acids (see Bentley and Housley, 1952, for a discussion on the production of acid in solutions of IAN in the presence of *Avena* coleoptiles). If IAN is converted into IAA by the plant material, and if the cells of the coleoptile are more permeable to IAN than to IAA, then it is conceivable that higher concentrations of acid could be produced from the nitrile within the cells than could be achieved by entry of the acid itself. This could explain the greater activity of the nitrile in promoting cell-elongation in the *Avena* straight-growth test.

4. Evidence for the Existence of the Nitrile in Radish Seedlings

It is of interest to consider earlier work on plant extracts, using the curvature test as a means of assay, in the light of results reported here. Stewart (1939) reports that there is an inhibitor in seedling plants of radish and other members of the Cruciferae family, on the basis of positive curvatures obtained in the curvature test. In view of results obtained with the nitrile in the curvature test, it was decided to investigate further the growth substances of radish.

Extracts of young radish seedlings (var. 'French Breakfast', the same as that used by Stewart) were prepared by two methods. The first method was that used by Stewart, and the same concentration as that of Stewart was prepared (5 g. fresh weight yielding 1 c.c. of test solution). The seedlings were 22 days old, with two leaves beyond the cotyledons. This extract was tested in the *Avena* straight-growth test, and caused complete inhibition. However, subsequent 10-fold dilutions up to 1,000-fold caused marked growth-promotion; a 100-fold dilution had approximately the same activity as a 0·1 mg./l. solution of IAA. The undiluted extract was also tested by the *Avena* deseeded method (Skoog, 1937) and usually caused positive curvatures after 5 hours. This result is similar to that obtained by Stewart. Furthermore, synthetic IAN (50 mg./l.), tested at the same time, also caused marked positive curvatures.

It has been noticed in this laboratory that coleoptiles deseeded by Skoog's technique have ceased to grow by the time they are ready for use in the assay;

positive curvatures therefore indicate that growth is occurring on the side of the coleoptile opposite the agar block and not that there is inhibition of growth on the side below the block. A true inhibitor would cause no response on deseeded coleoptiles. It has already been suggested in this paper that IAN may spread easily across the coleoptile, and this is confirmed by the positive curvatures obtained at high concentrations. Evidently the nitrile spreads across the coleoptile from the agar block and arrives at the other side in concentrations low enough to cause growth-promotion. If the neutral hormone in radish is the nitrile, then, from the amount of material used, it would probably be sufficiently concentrated in Stewart's extracts to cause positive curvatures, which would increase as more nitrile diffused from the block.

Also, the fact that Stewart obtained an increase in growth at the lowest dilution in straight-growth tests with intact coleoptiles, and negative curvatures in the earliest stages of curvature tests, shows a growth-promoting action of his extracts at low dilutions.

Extracts of further batches of seedlings, prepared by the same method, showed that at the four-leaf stage (29 days old) they contained considerably more auxin; a 100-fold dilution had approximately the same activity in the straight-growth test as a 1·0 mg./l. solution of IAA, that is, 10 times greater than the extract of 22-day-old seedlings.

A further extract (crushed tissue extracted for 20 hours at $-7°$) was prepared from 40-day-old plants, by Dr. S. Dunstan of the Chemistry Department. This extract showed activity in the straight-growth test greater than the maximum obtained with IAA, a strong indication of the presence of IAN.

Stewart's inhibitor is a neutral compound, its molecular weight is reported to be of the same order as that of IAA but lower, and it gives IAA on alkaline hydrolysis; it is also inactive in lateral bud inhibition. These are all properties of the nitrile. Stewart reports, however, that his substance is active in the pea test, and that it travels from base to apex in the coleoptile. These are not properties of the nitrile at physiological concentrations, but activity in the pea test is obtained at high concentrations (see Section 5). It has also been noticed that movement from base to apex in the coleoptile can occur at high concentrations (Table I, Expts. 43 and 59).

Thus it is suggested that the results reported by Stewart may be due, either entirely or in part, to high concentrations of a neutral hormone, probably IAN, in his extracts. It is proposed to investigate further the nature of the neutral hormone of radish and the possible existence of an inhibitor.

5. PEA CURVATURE TEST AND STRAIGHT-GROWTH OF PEA STEM SECTIONS

Indolylacetonitrile is inactive in the pea test (technique of Went, 1934, modified by Van Overbeek and Went, 1937) over a range of concentrations from 0·01 to 20–50 mg./l., the response of the split stems in IAN being identical with that in water. Veldstra (1944) also reports that IAN is inactive

in the pea test. There is, however, slight activity at concentrations above 20–50 mg./l. This result is interesting in view of the widespread use of the pea test as a method of measuring auxin activity without the interference of secondary factors such as transport, which exist in the *Avena* curvature test.

As previous workers have pointed out (summarized by Pincus and Thimann, 1948, chap. 2), the mechanism of the pea test does not appear to be a straightforward growth reaction. Etiolated pea stems, when split longitudinally, normally curve outwards, due to tissue tension. Under the influence of IAA, the two halves curve inwards, indicating greater growth of the outer tissues. (It has also been noticed in this laboratory that, if intact pea sections grown in solutions of IAA are subsequently split, the two halves do not curve outwards to the same extent as do those grown in water. This effect indicates a release of tissue tensions of the intact stem, possibly by an effect on suction pressure gradients.)

In any event, the reaction of split pea stems to IAA appears to be an unusual type of growth, and the possibility was considered that IAN might cause a different type of growth response in the pea test, e.g. growth in both inner and outer tissues such that there is no differential growth leading to inward curvature. However, the nitrile is also inactive in the straight-growth of intact pea sections at concentrations below 50 mg./l., although IAA is markedly active at concentrations as low as 1·0 mg./l. Thus it can be concluded that the nitrile has no effect on the growth of pea stems, except at high concentrations. The possibility that activity at high concentrations is due to production of the acid from the nitrile is being investigated, but if acid is produced, it must be less than 1 per cent. in 10 mg./l. solutions, on the assumption that it is IAA, since concentrations of IAA greater than 0·1 mg./l. cause activity in the pea test.

Preliminary experiments have been carried out to determine the activity (measured by the *Avena* straight-growth test) remaining in staled solutions of IAA and IAN from the pea test, and these show that, whereas there is a marked fall in activity with IAA, to below 50 per cent. of that of unstaled solutions, there is only a slight fall with IAN, the activity of which remains usually above 70 per cent. of that of unstaled solutions. These results are consistent with the suggestion that the acid is utilized or destroyed in the pea test (the auxin-inactivating enzyme in peas, investigated by Tang and Bonner, 1947, was shown to be specific for IAA), and that the nitrile is not affected.

These results raise several further questions. For example, it is necessary to know whether IAN is present in growing pea stems. Larsen (1944) has reported the presence of a neutral hormone in etiolated pea stems, and has suggested that it is 3-indolylacetaldehyde. Indolylacetaldehyde is active in the pea test at concentrations of 0·1–10·0 mg./l. (Bentley and Housley, 1952). The pea test, used in conjunction with the *Avena* straight-growth test, could therefore be used to differentiate between the aldehyde and the nitrile in neutral plant extracts.

Also, the high growth-promoting activity of the nitrile in *Avena* coleoptiles and its low activity in pea stems suggest investigation into the selective herbicidal effects of nitriles on monocotyledons and dicotyledons (see also the effect of IAN on root growth in cress and oats, Section 6).

6. INHIBITION OF ROOT GROWTH

Investigations on the effect of auxins on root growth have shown that, in general, substances which cause growth promotion in the *Avena* coleoptile cause inhibition of root growth. In a series of investigations on the effect of IAA on root growth, using Moewus's cress test (Moewus, 1949), one of us (Bentley, 1951) showed that IAA inhibits root growth in *Lepidium sativum* over a range of concentrations from 1·0 mg./l. to approximately 10^{-5} mg./l. under conditions in this laboratory. There is usually slight growth promotion at lower concentrations, to a maximum of about 30 per cent. at 10^{-7} to 10^{-8} mg./l., although this effect is not always obtained.

A similar investigation comparing the effects of IAA and IAN on root growth has now been carried out, using a modified form of Moewus's cress test, in which the substance to be tested is incorporated in 10 ml. of 1·5 per cent. agar in a small Petri dish 5 cm. in diameter. Less variable results are obtained with roots grown on agar than with roots grown on filter-paper (as used by Moewus), possibly because there may be absorption of the active substances on filter-paper, and because there is less tendency for roots growing on agar to grow up into the air, and so escape the influence of the substance being tested.

Results of a typical experiment (Fig. 3) show that IAN is approximately 100 times less active than IAA in inhibiting root growth in cress at concentrations from 0·05–50 mg./l. It is inactive below 0·05 mg./l. Results of further experiments are detailed in Table III. Variation throughout the tests is of the same order as illustrated in Fig. 3. It will be noted that a slight growth-promoting effect, of the same order as that obtained with IAA, is sometimes obtained with IAN below 0·05–0·005 mg./l. (Expts. 40 and 44), but the effect is as irregular as with IAA.

Thus IAN is inactive or slightly growth-promoting over a range of concentrations in which IAA inhibits root growth in cress. In view of this, it is necessary to determine which of the two hormones (if either) is operative in normal root growth, before interpretations in terms of the nitrile are possible. Warmke and Warmke (1950) have shown that there is a neutral auxin in roots of *Chicorium*, and it has been shown in this laboratory that there is considerably more neutral hormone than acid hormone in swede and turnip roots. On the Cholodny–Went theory of geotropism in roots, which postulates that geotropism occurs because of the inhibiting effect of hormone collecting on the lower side of horizontally laid roots, the concentration would need to be considerably higher to cause inhibition if it were the nitrile than if it were the acid. Similarly, the concentration of nitrile would have to be high to conform with current theories that the growth of the normal intact root is inhibited

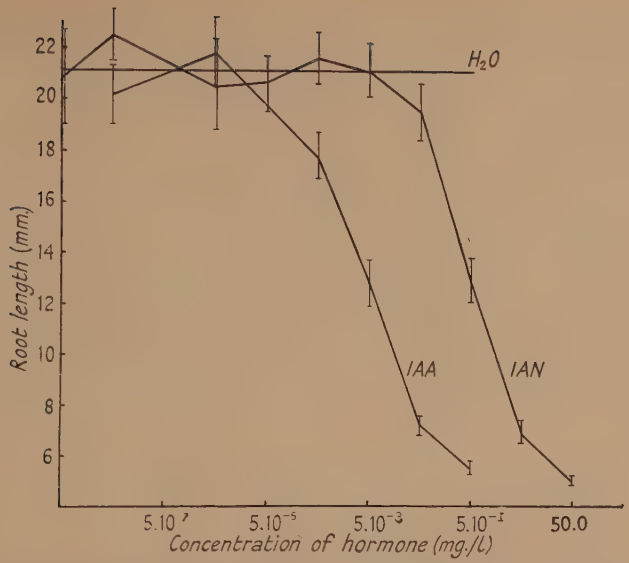

FIG. 3. Response of cress roots to IAA and IAN. Each point is the mean of 12 replicates (except water controls = 30 replicates). Vertical lines denote 2 × standard error at each point.

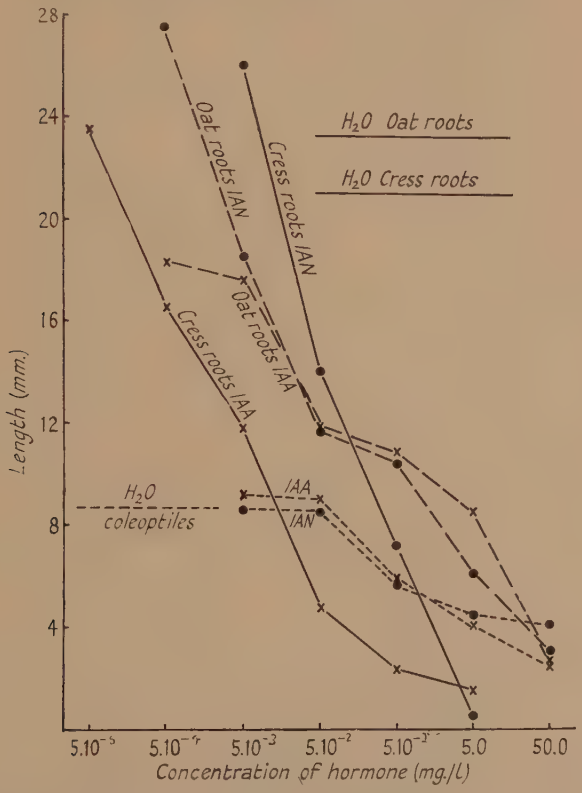

FIG. 4. Effect of IAA and IAN on germination in cress and oats.

Table III

Response of cress roots to IAA, IAN, and water

Figures denote final length of roots initially 5 mm. long. Bracketed figures denote numbers of replicates where these differ from column 2. Solution 1 (column 1) is 50 mg./l. and the following solutions are successive tenfold dilutions. NP denotes that the nitrile used was the natural product

Expt. No.	No. of replicates	Hormone	Concentration of hormone												H_2O
			1	2	3	4	5	6	7	8	9	10	11	12	13	
36 (NP)	6	IAA	—	—	—	2·3	7·5	14·2	17·5	—	17·9	—	18·0	—	11·1	17·4 (12)
		IAN	—	—	5·4	16·0	17·1	18·1	17·2	17·0	—	18·7	—	16·9	—	
40	6	IAA	—	3·1	5·1	6·2	10·6	11·0	18·2	—	—	—	—	—	—	14·7 (18)
		IAN	2·9	5·5	12·8	13·6	15·2	13·2	18·8	—	—	—	—	—	—	
41	12	IAA	—	—	6·5	7·8	11·8	17·7	18·4	20·0	—	20·5	18·6	18·1	—	20·3 (18)
		IAN	—	—	16·2	18·2	20·5	20·7	—	21·5	20·9	21·1	18·6	21·6	—	
42	12	IAA	—	—	5·4	7·1	12·8	17·7	—	21·7	—	20·1	—	—	—	21·1 (27)
		IAN	4·9	6·9	12·9	19·4	21·0	21·5	20·6	20·5	—	22·6	20·9	—	—	
44	12	IAA	—	5·4 (6)	5·3 (6)	7·3 (6)	10·7	19·6	22·5	18·2	21·4	22·2	20·3	—	—	20·2 (27)
		IAN	—	8·5 (6)	18·7 (6)	20·2 (6)	20·8	24·2	22·5	19·8	19·8	20·8	20·3	—	—	

because the hormone concentration comes within the inhibiting range. Further work on the occurrence and concentration of the nitrile in roots is indicated.

The effect of IAA and IAN on germination of cress and oat seeds on 1·5 per cent. agar (as measured by subsequent root growth) has also been studied. Ten seeds per test solution in 20 ml. of agar in a large Petri dish (9 cm. diameter) were allowed to germinate at 25°. The mean length per dish of cress roots was determined after 48 hours, and the mean length per dish of oat roots (the longest root per seedling was measured) after 72 hours. The results show (Fig. 4) that IAN is less active than IAA in inhibiting root growth in cress, but has approximately the same activity in oats, at 0·5–0·005 mg./l. The differential effect on cress and oats supports the suggestion put forward in Section 5 that nitriles might have a selective herbicidal effect on monocotyledons rather than on dicotyledons.

7. PARTHENOCARPIC FRUIT DEVELOPMENT

Solutions of the nitrile used in this and the remaining tests reported in this paper were prepared from concentrates of the naturally occurring substance, the exact hormone content of which was not known. The results can therefore only be regarded as preliminary, and the tests will be repeated with synthetic nitrile.

Experiments were carried out during the summer of 1951 on the effect of IAN on parthenocarpic fruit development in tomato (variety 'Potentate'). The technique of Luckwill (1948) was used, in which a constant volume of solution (in these experiments 0·03 c.c.) at a concentration of 100 mg./l. was applied by means of a hypodermic syringe to the ovaries of tomato flowers from which the stamens and styles had been removed. The solutions were applied at the stage when the petals were just unfolding. Two ovaries on a truss were treated, and the remaining flowers removed. In the first experiment, ovaries of the first truss were treated with β-naphthoxyacetic acid (BNOA), IAN, and water, rates of development were studied and final size determined. BNOA was used as control as it was known to be effective in causing parthenocarpy in tomato. Later, ovaries of the second truss on the same plants were treated with IAA in order to get some indication of the activity of IAA, although the results are not strictly comparable with those obtained with the first truss.

The results show (Table IV) that ovaries treated with BNOA grew at twice the rate, and to double the size of ovaries treated with IAN or IAA. Also, there was a long delay with IAN between application and beginning of development. This delay did not occur with BNOA or IAA, and therefore suggests that IAN may be inactive and may be changed over a period of weeks to some other active substance, possibly IAA.

A second experiment, designed to compare directly the effects of IAA and IAN, was carried out in July 1951. Ovaries on the first and second trusses of a new batch of plants were treated with IAA, IAN, and water in the same manner as in the first experiment. These plants developed very slowly,

because of the conditions under which they were grown, and only 20 per cent. of the hormone-treated ovaries developed. These attained an average diameter of 25 mm. with both IAA and IAN. None of the water-treated ovaries developed. The results are therefore qualitatively similar to those obtained in the first experiment.

TABLE IV

The effect of various hormones on parthenocarpic fruit development in tomato (22 ovaries per treatment)

Substance	Average growth rate of ovaries (mm./day)	Final diameter of ovaries (mm.)	% ovaries developed	Date of treatment (June 1951)	Date development of ovaries began
BNOA	1·5	70	73	2–11	Immediately
IAN	0·6	35	68	2–11	After 21 days
IAA	0·7	42	36	19	Immediately
H_2O	—	2–4	—	2–11	—

Thus IAN shows no greater activity than IAA in inducing parthenocarpic fruit development, and this activity is far below that shown by a synthetic growth-substance, β-naphthoxyacetic acid. Yet experiments in this laboratory have shown that, in *Avena* straight-growth tests, the latter substance has only slight activity, of less than 1 per cent. that of IAA. The contrast between the activities of these substances in the two tests suggests that the mechanism of enlargement in developing fruits follows a different system from that effective in cell-elongation in *Avena* coleoptiles.

As a matter of interest it was noticed during the experiments that, although ovaries treated with water usually failed to develop, four ovaries developed to an average diameter of 20 mm. All these were on trusses below ones which had been treated with 2:4-dichlorophenoxyacetic acid for the purposes of another experiment. There would thus appear to be some transmission of the stimulus for fruit development through the plant.

8. LATERAL BUD INHIBITION

Young plants of *Phaseolus multiflorus* were decapitated when the internode above the first two leaves was approximately 6–8 cm. long. The internode was cut to a length of 2 cm. above the node, and a glass tube was fixed to the cut end by means of vaseline or porometer paste to make a watertight join. The porometer paste was prepared from colophony resin, vaseline, and small pieces of rubber tubing. A volume of 0·2–0·3 c.c. of test solution was then pipetted in. By this technique it was possible to get complete inhibition of lateral bud development with IAA at 20 mg./l. The solution of the nitrile (natural product) was also approximately 20 mg./l. The solutions were renewed every day, any remaining in the tubes being pipetted out and tested for activity in the straight-growth test. Development of the buds in the axils of the first two leaves was studied for 6–7 days.

The results show (Fig. 5) that the nitrile, at the concentration tested, is inactive in inhibiting lateral bud development. Solutions removed from the tubes were fully active in the straight-growth test, therefore there had not been destruction of the nitrile. Failure to inhibit bud development may be because the nitrile is not transported down the shoot, although it has been

FIG. 5. Effect of IAA, IAN (labelled C 436) and water on lateral bud development in *Phaseolus multiflorus*.

shown earlier in this paper that it is easily transported in the *Avena* coleoptile. Alternatively, it may be inactive. Furthermore, the experiments give no evidence of hydrolysis to the acid, since the latter is active in bud inhibition.

9. INITIATION OF CAMBIAL ACTIVITY

Söding (1936) and Gouwentak (1941) have shown that if IAA is applied to resting twigs of *Salix* spp., there is initiation of cambial activity below the point of application, with the production of a new ring of wood. Both these workers used relatively high initial concentrations of hormone, the former using pure crystalline IAA and the latter using 0·1 mg. IAA applied in lanoline. The technique was adapted in the experiments reported here for use with low concentrations of hormone.

Resting twigs of *Salix* spp. were gathered in early March before the buds began to unfold, and before cambial activity had commenced. They were debudded and stored at 0° until required for use. Sections of twigs, usually about 10–15 cm. long, were selected, and glass tubes of the same bore as the twigs were fixed to the upper cut surfaces with adhesive tape and patent rubber solution to ensure watertight joins. Approximately 0·5 c.c. test solution was pipetted into each tube. The solutions were renewed every day

except at the beginning of the experiment, when uptake of solution was rapi and it was necessary to renew twice in the first day. The twigs were place with their lower ends in distilled water, which was renewed every week.

Results of a typical experiment (20 mg./l. hormone solutions applied fo 12 days from 23 May) show (Fig. 6) that there is initiation of cambial activit and production of a new ring of wood ½ cm. below the cut surface to approx mately the same degree with both IAA and IAN. There is no activity i water-treated twigs. Sectioning was continued down the twigs, and sligh

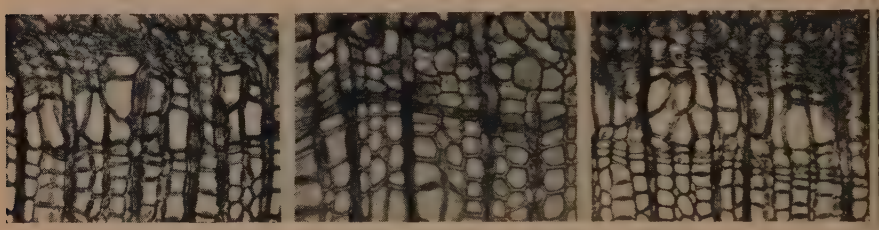

FIG. 6. Production of new wood in twigs of *Salix* treated with hormone solutions. Left, IA (natural product); middle, water; right, IAA. The sections were cut ½ cm. below point c application of the solutions.

activity could be detected as far as 5 cm. below the treated surface. Simila results are being obtained from experiments at present in progress (1952 with synthetic nitrile at 20 mg./l.

Preliminary experiments were carried out with IAA alone, before IAN wa tested, to get some idea of the concentrations required for activity. The result present one or two interesting points and will be briefly reported.

In the first experiment (2 mg./l. IAA applied for 36 days from 19 March there was slight activity with irregular production of new wood just belo the upper cut surface, even at this low concentration. In the second experi ment (commenced 1 May for 14 days) there was production of new woo along the whole length of the twig at concentrations of both 20 mg./l. an 5 mg./l. IAA. The new ring of wood was widest in the upper 2 cm. an diminished to a layer only one cell wide at a distance of 14–15 cm. In a late experiment (18 June for 14 days) activity of both the acid and the nitrile a 20 mg./l. was much lower than in earlier experiments and extended onl 1–2 cm. down the twigs. The twigs were then 3½ months old and were possibl not as sensitive to applied hormone as at an earlier date.

Thus IAN is active in causing initiation of cambial activity and the pro duction of new wood in willow to the same extent as IAA.

10. ROOT-INITIATION, AND INHIBITION OF PETIOLE ABSCISSION

Experiments on root-initiation in *Phaseolus vulgaris* (technique of Mitche and Stuart, 1938) were carried out in June and July 1951 with solutions o the naturally occurring nitrile at a concentration of approximately 20 mg./

at this concentration IAA is markedly active, but the nitrile showed only very slight activity, which was not significantly greater than that of water. In preliminary experiments with synthetic nitrile at concentrations above 20 mg./l., at present in progress (March 1952), there is activity greater than that of water but still markedly less than that of IAA. Also, the response to nitrile is delayed in a manner similar to that in parthenocarpic fruit development (Section 7), so that the roots developing on the nitrile-treated plants are shorter than those on the acid-treated plants. These experiments will be continued.

Tests on the effect of IAA and IAN on petiole abscission in apple were kindly carried out in the autumn of 1951 by Mr. H. W. B. Barlow of East Malling Research Station, Kent, using his previously reported technique (Barlow, 1950). He reports that IAN (natural product) was inactive or only slightly active in inhibiting petiole abscission, over a range of concentrations from approximately 1·0–100 mg. l., whereas IAA showed definite activity at concentrations from 25–100 mg./l.

The solutions of the naturally occurring nitrile used in the experiments on rooting and petiole abscission were highly active in the *Avena* straight-growth test, both having activities greater than those of IAA at comparable dilutions from 20–0·1 mg./l. Therefore the low responses obtained are not due to loss of activity in the solutions.

DISCUSSION

Table V gives a summary of the relative activities of IAA and IAN in the various aspects of growth and development examined in this paper. The significance of these results is discussed to some extent in the individual sections, but in addition it is now possible to consider wider aspects of the activity of the nitrile as a growth hormone.

It is seen from the table that, apart from results with *Avena*, which is the only monocotyledon among the species tested, the nitrile is as active as the acid only in cambial initiation in *Salix* and parthenocarpic fruit development in tomato. (It must be remembered that in the latter test there was a marked delay between application and swelling of the ovaries, whereas ovaries treated with the acid began to develop immediately.) In all the other tests the nitrile is either less active than the acid, or inactive at the concentrations tested. If the nitrile is active by reason of its conversion to a second substance, e.g. IAA, then only *Salix* and *Avena* of the species tested can achieve this conversion efficiently. It would clearly be desirable to examine the metabolism of the nitrile in the various tissues concerned.

Alternatively, the nitrile and the acid may both influence various aspects of growth independently. If this is so, it should be noted that the nitrile is as active as the acid in tests affecting cell-division in meristematic tissues (e.g. cambial initiation, fruit development), and does not affect or has lower activity than the acid in those aspects of development which do not usually occur in

TABLE V

Relative activities of IAA and IAN in various aspects of growth and developmen

Growth phenomena	Concentrations of hormone (mg./l.)	IAA	IAN	Degree of activity
1. Straight-growth of *Avena* sections	0·001–100	+	+	Nitrile > Acid
2. Curvature in standard *Avena* test	0·01–0·1	+	+	Nitrile ≏ Acid
	0·1–1·0	+	+	Nitrile < Acid
3. Polar transport from apex to base in *Avena* coleoptile	0·05–0·5	+	+	Nitrile ≏ Acid
4. Transport from base to apex in *Avena* coleoptile	0·05–0·5	−	−	—
5. Curvature of split pea stems	0·1–20	+	−	Nitrile slightly ac tive at higher con centrations
6. Inhibition of root-growth:				
(*a*) in cress	$5 \cdot 10^{-5}$–50	+	+	Nitrile < Acid
(*b*) in oats	$5 \cdot 10^{-3}$–50	+	+	Nitrile ≏ Acid
7. Inhibition of bud-growth in *Phaseolus multiflorus*	~ 20	+	−	—
8. Inhibition of petiole-abscission in apple	~ 1·0–100	+	−	Nitrile slightly ac tive at 100 mg./l.
9. Initiation of cambial activity in *Salix* sp.	~ 20	+	+	Nitrile ≏ Acid
10. Root-initiation in *Phaseolus vulgaris*	~ 20	+	−	Nitrile sometime showed slight de layed activity
11. Parthenocarpic fruit development in tomato	~ 100	+	+	Nitrile ≏ Acid but delayed

such tissues (e.g. lateral bud inhibition, root initiation, inhibition of roo elongation, petiole abscission). These facts would suggest that the nitril may be operative as a hormone in meristematic tissues only, whereas the aci may be operative in aspects of development originating in older tissues Obviously, to test this hypothesis, it would be necessary to determine th distribution of the two substances in the types of tissue used in the variou tests. This could be done by paper partition chromatography, as it has bee shown in the Chemistry Department of this University that the acid and th nitrile are easily separable by paper chromatography. Also Bennet-Clark *et a* (1952) and Luckwill (1952) have shown that it is possible to obtain clea separation of growth-promoting and inhibiting substances in extracts fro range of plant material by paper partition chromatography.

Further results in the various tests described here will be reported i subsequent papers in this series.

The authors have pleasure in repeating the acknowledgements made in th first paper of this series.

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Studies in Stomatal Behaviour

II. THE ROLE OF STARCH IN THE LIGHT RESPONSE OF STOMATA

Part 4. Variation under Constant Conditions

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SUMMARY

A quantitative study has been made of the extent and nature of the variation in starch-content and aperture of the stomata of *Pelargonium*. It is shown that distribution over the surface of a single leaf is substantially normal in form; that under experimental conditions in common use there is a highly significant difference between groups of stomata on the lower epidermis of a single leaf; that the variation between different leaves on the same plant, or between similar leaves on different plants, does not differ significantly from that between strips from the same leaf; that not less than 20 stomata should be measured on each strip; and that pore-width is a satisfactory measure of the area of the stomatal aperture.

INTRODUCTION

The previous paper in this series (Williams, 1952) presented information o the effect of external factors on the starch-content of guard-cells, investigate by a modification of the quantitative technique of Williams and Spencer (1950 The results were examined by the now conventional technique of the analys of variance; but the use of such analysis implies that the data may be regarde as drawn from a continuously varying population of substantially 'norma distribution. Since the data are of a new type this assumption requires just fication; but, since the evidence in its support is inevitably both bulky an limited in appeal, I have thought it appropriate that it should be segregate into this separate communication.

The methods of measurement were fully described and discussed in th previous paper, and call for no further comment; all data refer as usual t attached leaves of *Pelargonium zonale* var. 'Paul Crampel'.

I. THE NATURE OF THE VARIATION

1. *Variation over a single leaf*

(*a*) *Between individual stomata.* A leaf was illuminated for 1 hour (from 09 to 1000 hours G.M.T. in May) by means of a 150-watt water-cooled incan descent lamp; a ground-glass screen was interposed between the lamp and th leaf to increase the evenness of illumination. The leaf was freely exposed the air of the laboratory. At the end of the experimental period six strips epidermis were taken from both the upper and lower surfaces (see below f

a note as to the positions from which the strips were removed), and the starch and aperture of 20 stomata on each strip measured in the usual way. The results may thus be regarded as four populations: upper starch, upper aperture, lower starch, and lower aperture, each of 120 members. Histograms of these populations (grouped, with $10\mu^2$ as class-interval) are shown in Fig. 1;

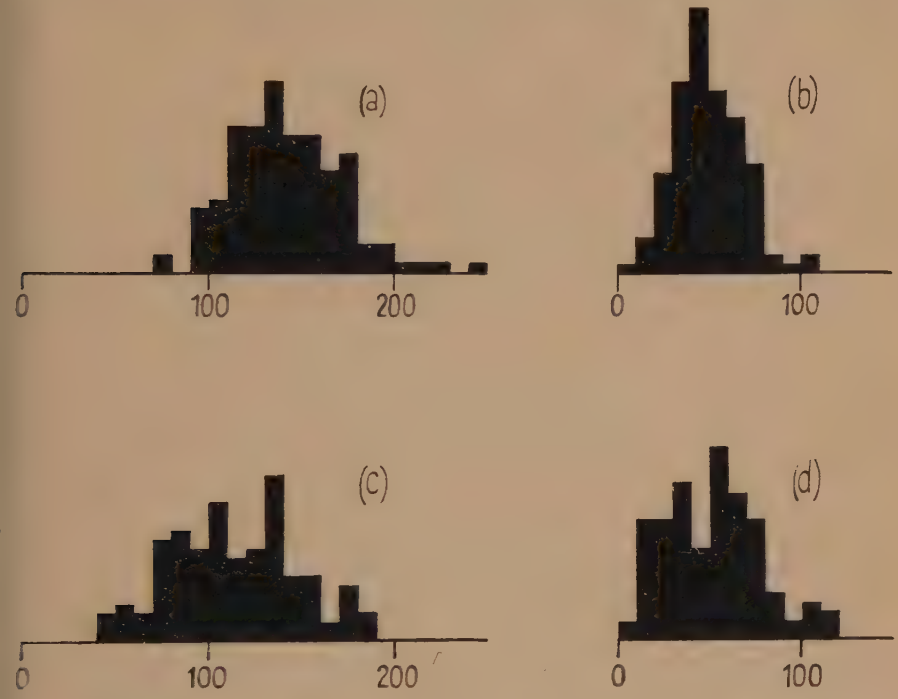

FIG. 1. Distribution of starch and aperture over a single leaf. (*a*) upper starch, (*b*) upper aperture, (*c*) lower starch, (*d*) lower aperture. Values are in μ^2.

means, standard deviations, and Pearson coefficients have been calculated from the grouped data and are given in Table I (for the normal distribution $\beta_1 = 0$ and $\beta_2 = 3$). It is immediately clear that the distributions are substantially symmetrical and of normal kurtosis; the techniques of the analysis of variance may therefore be applied with confidence.

TABLE I

Statistical data: variation over a single leaf

	Upper epidermis		Lower epidermis	
	Starch	Aperture	Starch	Aperture
$\bar{x}$	141·00	49·67	113·92	51·75
s	30·32	18·61	33·82	25·86
β_1	0·28	0·14	0·01	0·14
β_2	3·54	3·25	2·37	2·61
F	1·87	0·39	5·30	7·82

(F is here the ratio of the variances 'between' and 'within' strips.)

Calculation of a correlation coefficient from the data coarsely grouped into class-intervals of $20\mu^2$ suggests the presence of a small, and probably non-significant, positive correlation. Greater reliance may probably be placed on the highly significant value of *c.* +0·5 reported in the previous paper, since this was based on ungrouped data with 304 degrees of freedom.

(*b*) *Between strips.* An unexpected feature of the histograms in Fig. 1 is the slightly greater 'spread', and considerably greater irregularity, of the data for the lower epidermis; and it is of considerable practical importance to ascertain whether this is due to variation between individual stomata, or to a high variance between strips (*scil.*, groups of stomata). A simple analysis of variance into 'between strips' and 'within strips' has therefore been carried out on each population, and the values of the variance ratio F (between/within, with 5 and 114 D.F. respectively) are also given in Table I. F does not attain significance for either set of upper epidermal data; but it is significant at the 0·001 probability level for both lower sets. Evidently an appreciable part of the increased variation of the lower epidermis lies between, rather than within, strips.

This variation is not connected with specific positions on the leaf. The six strips were selected to represent different positions (i) laterally from the midrib, and (ii) inwards from the margin; in other words, if the leaf be regarded as a figure in polar co-ordinates with the origin at the junction of the petiole and lamina, attempts were made to vary both θ and r within the limits of the lamina. No corresponding regularity could be observed in the results. It seems likely, therefore, that the variation is due to small differences in light intensity and humidity over the lower surface of the leaf; the causes could no doubt be elucidated by further work, but it is doubtful whether the results could justify the effort involved. It may be said in conclusion that the choice of residual 'between strips' variance as error in the previous paper was evidently the correct procedure; a true 'within strips' error variance would have been misleadingly low.

2. *Variation between leaves*

Seven plants were selected, as alike in size and vigour as possible. It being manifestly impracticable to illuminate all leaves of these plants equally, the plants were allowed to remain for an hour (1400 to 1500 hours G.M.T. in May) in the shade under a bench in the greenhouse; by this means they were exposed to a substantially even, though low, intensity of diffused daylight. At the end of the experimental period stripping was carried out as follows:

(*a*) From one plant a single strip of lower epidermis was removed from a comparable position on every expanded leaf, including two immature leaves of an axillary bud—nine strips in all.

(*b*) From each of the remaining six plants a single strip of lower epidermis was taken from one leaf; the leaf selected was in each case the first fully expanded and freely exposed leaf on the plant—usually the third.

After measurement of 20 stomata on each strip, 2 sets of results were thus obtained: a set of 9 values of starch and aperture for different leaves of the same plant, and a set of 6 values for similar leaves of different plants. It is unfortunate that further strips were not taken from one of these leaves, since the information on variation between strips on any one leaf already obtained is referable to somewhat different external conditions; however, in the absence of more suitable data, this information will be treated as a comparable measure of variation.

The variances are given in Table II. In the case of both starch and aperture, the ratio of the largest to the smallest variance fails to reach significance, and no useful purpose would be served by applying Bartlett's test (as had originally been intended) to all three variances. Two points merit brief mention:

(i) *Starch.* The ratio of 'similar leaves of different plants' to 'within a leaf' comes close to significance

$$(F = 4{\cdot}5;\ \text{for } P = 0{\cdot}05 \text{ with } n_1 = n_2 = 5,\ F = 5{\cdot}1).$$

It is therefore likely that occasional experiments will show a significant difference between leaves; but the highly significant difference noted in Expt. 1 (but not in other experiments) of the previous communication now seems likely to have been brought about by the different order of treatments received by the two pairs of plants rather than by any inherent difference between the leaves concerned.

(ii) *Aperture.* The variances for aperture are in the reverse of the expected order—the highest within a single leaf, the lowest between leaves of different plants. However, the low variance ratio ($F = 2{\cdot}9$, where significance at the level $P = 0{\cdot}05$ would again require $F = 5{\cdot}1$) suggests that the result may well be entirely fortuitous.

TABLE II

Comparison of variances (calculated on strip means)

	Starch	Aperture
Different strips, same leaf . .	247·58	203·22
Different leaves, same plant . .	330·54	117·32
Similar leaves, different plants .	1,122·86	70·47

II. EXAMINATION OF TECHNIQUES

1. *Sample size*

Throughout this investigation a sample of 20 stomata has been taken from each strip. The measurement of even this small number is extremely tedious, and if it could be reduced without serious error the method would be more useful. Owing to the erratic variation between groups of stomata it seemed desirable to investigate this possibility by direct observation rather than by invoking statistical inference. The results for 10 strips were therefore re-examined: the mean values for starch and aperture calculated from the first 10 stomata drawn were compared with the values obtained from all 20.

Although agreement was in some cases extremely close, the errors in other amounted, in starch or aperture, to as much as 14 per cent.; it therefore seem inadvisable to take a sample of less than 20 stomata per strip.

2. Pore-width as a measure of aperture

When Lloyd's stripping technique is used for stomatal examination a estimate of the mean area of stomatal aperture is normally required. Tw methods have been employed at various times: first, both length and breadt (at the widest point) are measured and the area calculated on the assumptio that the area is accurately elliptical; secondly, breadth alone is measured, being assumed that length remains substantially constant during movement The validity of both these assumptions can be checked on the drawing obtained in the course of the present investigation.

A leaf was illuminated and part enclosed between glass strips for 2 hours s that the stomata thus enclosed opened widely; a strip of lower epidermis wa then taken from both the enclosed and unenclosed areas, and 20 stomat drawn from each strip. Records were thus obtained of 40 stomata covering wide range of aperture; in each case the length, breadth (at the widest point) and area (by planimeter) were measured and the results reduced to μ or μ as appropriate.

(*a*) *Calculated area.* Fig. 2(*a*) is a scatter-diagram in which the abscis represents actual (measured) area, the ordinate area calculated from th formula (area $= \pi ab$), where a and b are the semi-axes: the line ($y = x$) ha been drawn in for comparison. It is clear that the approximation is remarkabl good; the regression equation of calculated on measured area is in fact

$$y = 1{\cdot}005x + 2{\cdot}845.$$

The regression coefficient has S.E. = 0·0253, is highly significant, and doe not differ significantly from unity.

(*b*) *Breadth.* We may first compare the variability of length and breadth i the 40 stomata. The statistics are:

Length:	mean	16·80
	S.E. of mean. . . .	0·244
	coefficient of variation . .	9·12
Breadth:	mean	6·04
	S.E. of mean. . . .	0·480
	coefficient of variation . .	50·28

The coefficients of variation ($100s/\bar{x}$), being dimensionless, provide the bes comparison and it is evidently true that breadth is more variable than length To compare breadth with area it is desirable to bring the two sets of measure ments to the same scale; the breadths have therefore been multiplied by factor (12·60) which brings the grand means of breadth and measured area int coincidence. Fig. 2 (*b*) shows the breadth, thus adjusted, plotted agains measured area with the line ($y = x$) drawn in; there seems little loss of accurac

in comparison with Fig. 2(*a*). The statistical relationships between breadth and area are as follows:

(i) Correlation coefficient: 0·9562 ($n = 38$).

(ii) Regression of adjusted breadth on measured area:

$$y = 0{\cdot}963x + 2{\cdot}685.$$

(iii) Regression of measured area on adjusted breadth:

$$x = 0{\cdot}949y + 3{\cdot}876.$$

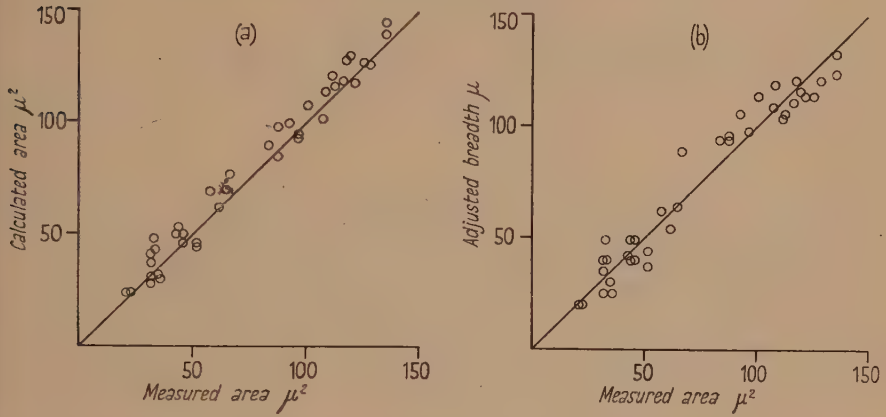

FIG. 2. Comparison of (*a*) calculated area, and (*b*) breadth, with measured area. The diagonal line is ($y = x$) in each case, not a regression line.

The S.E.s of the regression coefficients are (ii) 0·0478 and (iii) 0·0541; both coefficients are significant and neither differs significantly from unity.

The S.E. of the coefficient for breadth on measured area (ii) is about double that of the corresponding coefficient for calculated area on measured area, so there would evidently be a slight loss of accuracy if only very few stomata were measured; but it seems clear that, with 40 stomata, breadth provides an entirely adequate estimate of area; and the graphs suggest that this number might be reduced to, say, 20 without serious loss of accuracy.

ACKNOWLEDGEMENTS

The data on which this paper is based were collected while I was still a member of the staff of Bedford College, London, and I am indebted to Professor L. J. Audus for permission to utilize them for this analysis. Once more I wish to express my grateful acknowledgements to Mr. J. C. Funnell, for his invaluable assistance in the drawing and planimeter measurements; to Mr. G. S. Spencer for permission to incorporate results obtained during our preliminary studies; and to Mr. G. Butler for provision of the *Pelargonium* plants.

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Factors controlling Flowering in the Chrysanthemum

III. FAVOURABLE EFFECTS OF LIMITED PERIODS OF LONG DAY ON INFLORESCENCE INITIATION

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Summary

Experiments are described which indicate that Chrysanthemum cuttings derived from unvernalized long-day stock plants flower sooner and with lower leaf numbers after vernalization than similarly treated cuttings from short-day stock. Long-day treatment of young cuttings also hastens inflorescence initiation provided the period of such treatment is limited and given before or immediately after vernalization. The effect of long day appears to be maximal when vernalization is complete. Long-day treatment cannot substitute for vernalization.

Introduction

Nearly all experiments published on the photoperiodism of the Chrysanthemum appear to have been carried out with vernalized plant material, a fact which was pointed out in the first paper of this series (Schwabe, 1950). In most cases these plants had also been maintained for some period in long-day conditions prior to their experimental treatments; and it seems not unlikely that this long-day (L.D.) pre-treatment might have influenced the subsequent behaviour of the plants to some extent. In the earlier paper, when discussing the vernalization requirement of the Chrysanthemum, it was mentioned that L.D. treatment might possibly serve as a partial substitute for exposure to low temperature. Such an effect would be analogous to short-day (S.D.) induction of winter rye which in some degree is effective in replacing low-temperature treatment (Purvis and Gregory, 1937). Chouard (1949) mentions that exposure of young Chrysanthemum cuttings to L.D. hastened their flowering relative to those given S.D. throughout. Finally, a pronounced effect of 'parentage' had been observed in an early experiment here where plants derived from L.D. and S.D. stocks had been compared under different conditions of vernalization and length of day (cf. Table I below). In an attempt to clarify these relations the experiments to be described were undertaken.

Methods and Results

The general methods of treating the plant material were identical with those described previously. Short-day treatment consisted of 8 hours' full light and

16 hours' darkness ; long-day treatment of 16·5 hours' light (of which 8 hours were full daylight) followed by 7·5 hours' dark. Effects on flowering behaviour were again estimated from leaf-number data and the time required for the production of the macroscopically visible flower bud. The heights of the plants were also measured in several of the experiments at the time when the plants became macroscopically budded or earlier as stated in the text.

In the presentation of the results it has not been possible in all cases to give significant differences ; and standard errors for the individual means are shown instead in Table III (*b*) and (*c*). This was found necessary owing to the unequal error distribution in experiments involving different degrees of vernalization. To ensure a more normal error distribution the logarithmic transformation was employed in the analysis of the data shown in Fig. 1 and Table IV (*b*).

1. '*Parentage effects.*' In an early experiment of 1948–9, in which vernalization and its absence were combined factorially with two lengths of day, one half of the plants was derived from L.D. and the other from S.D. parent stock. Since parentage appeared to have no effect in the unvernalized set, leaf numbers and times to flowering are presented in Table I for the vernalized set alone. Eight replicates were grown in each treatment, but the data for the continuous L.D. treatment are derived only from those plants which actually initiated inflorescence buds, i.e. 6/8 from L.D. parents and 3/8 from S.D. parents.

TABLE I

Effect of L.D. and S.D. parentage. Variety 'Indian Summer'

Final leaf number to flowering			Days to flowering after start of experiment		
	Day-length received			Day-length received	
Parentage	S.D.	L.D.	Parentage	S.D.	L.D.
L.D. . . .	25·6	43·0	L.D. . .	75·4	133·2
S.D. . . .	31·5	46·3	S.D. . .	86·4	176·3

Though the effect was not large, there appeared to be a definite reduction in both leaf number and time to flowering in the set derived from L.D. parents. To confirm this result a further experiment was set up in the following season, again combining L.D. and S.D. parentage with the same two day-lengths given during and after vernalization. The results are shown in Table II ; they confirm that L.D. parentage causes a highly significant reduction in leaf number and time to budding, which in the case of leaf numbers even exceeds the effect of the subsequent day-length given, though absolutely, of course, these reductions are not very large. Continuous L.D. is again seen to have a delaying effect on flower initiation.

2. *Interaction with vernalization.* The fact that the flowering of unvernalized plants did not appear to be hastened by exposure to L.D. was confirmed in a preliminary experiment. Some plants derived from L.D. parents and

TABLE II

'Parentage effect'. Experiment started 18.10.49. Variety 'Indian Summer', 10 replicates

		Leaf-number increment	Days to flowering after end of vernalization
Parentage	S.D.	24·7	101·4
	L.D.	21·8	92·3
Day-length received	S.D.	22·3	87·3
	L.D.	24·3	106·4

Significant differences (0·1 % level), 1·61 leaves; 6·06 days.

grown in L.D. for several weeks were transferred to S.D., while others were maintained in L.D. Only one of the three replicates transferred to S.D. initiated any inflorescences at all with a high leaf number (74) and more than 11 weeks after transfer. When the experiment was ended after 15 weeks the mean leaf number of the plants kept in L.D. (non-flowering) was 63, the other two completely vegetative plants in S.D. having produced an average of 70 leaves.

In a subsequent experiment three periods of vernalization were combined with three periods of L.D. given immediately after vernalization. All plants were from S.D. parent stock, and they were returned to S.D. after the L.D. treatment. The times of vernalization were 0, 2, and 4 weeks, the L.D. periods 0, 6 and 12 weeks. Each treatment was replicated 6 times. The results are shown in Table III.

TABLE III

Interaction of degree of vernalization with different L.D. periods. Experiment started 28.12.50, ended after 240 days. Variety 'Indian Summer', 6 replicates

	Weeks of long day	Weeks of vernalization		
		0	2	4
(*a*) Number of plants budded at end of experiment.	0	5	6	6
	6	4	6	6
	12	2	6	5*
(*b*) Leaf-number increment to budding. (Increment for vegetative plants in brackets.)	0	53·1±6·9 (70·0)	33·8±4·09	22·8±0·90
	6	48·7±7·7 (69·0)	37·6±4·09	18·5±0·90
	12	58·8±10·9 (64·0)	28·0±4·09	28·1±0·99
(*c*) Days to budding from start of experiment. Days from end of vernalization period are shown in brackets.	0	183±18·9	118(104)±8·8	102(74)±1·99
	6	134±21·1	125(111)±8·8	93(60)±1·99
	12	167±29·9	119(105)±8·8	119(91)±2·18
(*d*) Heights of plants (cm.) above soil level at time of budding. Sig. diff. = 7·3 cm.	0	37·0	24·7	25·3
	6	37·3	30·7	31·9
	12	47·3	56·2	73·0

* One plant died.

Owing to the fact that the variability between replicates is high in all unvernalized or partly vernalized treatments—as pointed out previously—the error values are high for the experiment as a whole. If a pooled error is used only the main effects reach significance; hence in Table III (*b*) and (*c*) the standard errors have been derived separately for the three vernalization sets. The difference between the controls (S.D. throughout) and those having had 6 weeks of L.D. is clear in the fully vernalized set. But while 6 weeks of L.D. speeded up the process of flower initiation, 12 weeks of L.D. retarded it as compared with the controls. The same holds also for the leaf-number data. In the partly vernalized group (2 weeks cold) and the unvernalized controls, leaf numbers and times to flowering do not differ significantly from the controls. The differences between the mean values for the three vernalization treatments are, of course, highly significant.

An analysis of the final heights of the budded plants also shows an interesting and highly significant interaction. The main effect of extensive L.D. treatment in increasing plant heights is seen to be much enhanced with increasing amounts of vernalization. The effect of this interaction on plant heights therefore serves to emphasize again the probable importance of both factors on the plants' auxin metabolism.

In a third experiment a fixed period of L.D. (4 weeks) was given after 0, 1, 2, 3, and 4 weeks' vernalization; control treatments for each time of vernalization were kept in S.D. throughout. All treatments were replicated six times. Unfortunately in one treatment (viz. 1 week's vernalization followed by L.D.) two very aberrant plants occurred; hence in Table IV and in Fig. 1 two means are shown (the value including these aberrant replicates in brackets). The logs of the final leaf numbers attained at flowering are shown in Fig. 1 and the times to budding in Table IV (*a*).

TABLE IV

Effect of 4 weeks' L.D. given after different periods of vernalization. Variety 'Indian Summer'. Experiment started 18.10.49; 6 replicates

	Day-length treatment	Weeks of vernalization 0	1	2	3	4	Mean
(*a*) Days to budding after end of vernalization.	S.D. control	193	169	123	95	87	133·6
	4 weeks' L.D.	190	167 (188)	104	84	76	124·3
						Sig. diff.	4·46
							Mean log
(*b*) Plant heights (cm.) 14 weeks after start.	S.D. control	7·1	7·7	12·8	19·3	20·3	1·081
	4 weeks' L.D.	7·7	7·5	17·2	24·5	23·5	1·139
						Sig. diff.	0·0495
(*c*) Plant heights at budding (cm.).	S.D. control	33·7	30·2	28·0	28·5	26·7	
	4 weeks' L.D.	32·8	34·5	24·4	26·8	25·4	

The highly significant main effect of 4 weeks' L.D. in hastening flower initiation is again seen clearly. The interaction with degree of vernalization is not significant in the analysis of the time to budding (due to the high

errors of the partially vernalized sets). But analysis of the leaf-number increments after log transformation indicates that this interaction is highly significant, and since this result also confirms a similar effect in the previous experiment (Table III (*b*)), it is very probable that the L.D. treatment is mainly effective only in conjunction with a minimum cold treatment.

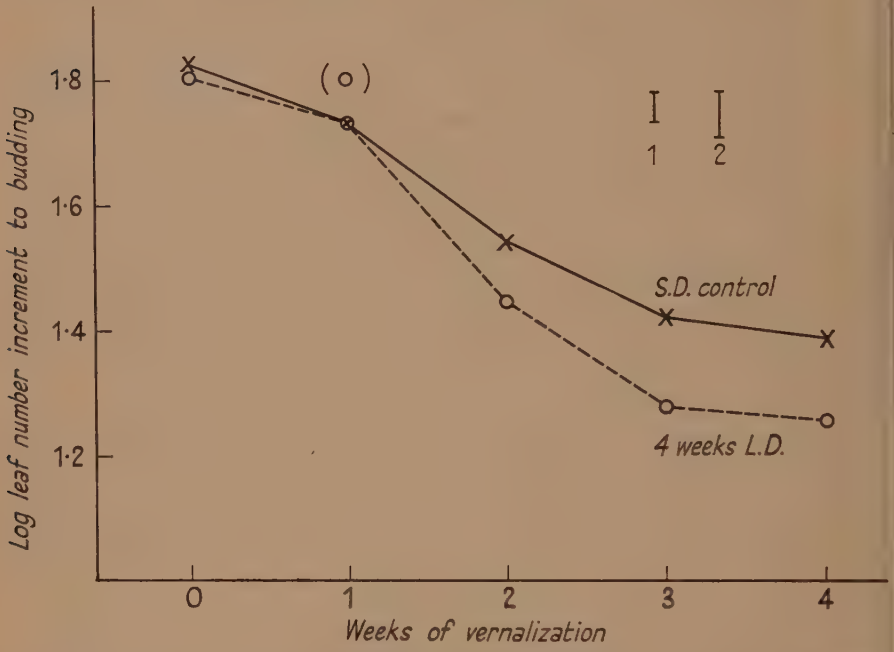

FIG. 1. Effect on log leaf-number increment to budding of 4 weeks' L.D. after different periods of vernalization. Two significant differences ($P = 0{\cdot}05$) are shown: based on an analysis (1) omitting two aberrant plants, (2) including all plants.

The total heights of the plants above soil level were also measured 14 weeks after the start of the experiment. The height measurements were repeated on each plant at the time of budding, and it is interesting to compare these figures. Initially the effect of 4 weeks' L.D. treatment is to increase plant height significantly above that of the S.D. controls, when summed over all treatments. But this effect is eliminated again by the time of budding; earlier budding due to the L.D. treatment has caused a slight though not significant reduction in height at that time of the L.D.-treated set relative to the S.D. controls.

The highly significant main effect of vernalization in increasing plant height (Table IV (*b*)) is due largely to increased internode length, but at the time of budding (Table IV (*c*)) total plant heights are smaller in the fully vernalized set owing to the much greater number of internodes produced in the partly vernalized treatments before budding.

The last experiment to be described was designed to discover at what time

the L.D. treatment must be applied to be effective. In this experiment again a fixed period of L.D. (4 weeks) was given after full vernalization (4 weeks), and a pre-vernalization treatment was also included to test whether the 'parentage' effect noted above could be ascribed to the L.D. treatment received by the cuttings on the parent plant prior to their re-rooting. The times of exposure to L.D. were as follows: L1 immediately before vernalization, L2 immediately after, L3 four weeks after, and L4 eight weeks after the end of vernalization. In addition there was a control set (S.D.) which received no L.D. treatment. When the plants started to form their inflorescence buds it was seen that the last L.D. treatment (8 weeks after vernalization) was given too late to have any effect, as the plants were just becoming budded at the time transfer to L.D. was due. The results are, however, included although their treatment prior to budding was identical with that of the S.D. controls, for they serve to indicate the precision which it is possible to attain with fully vernalized plants. The results shown in Table V demonstrate once more the hastening of flowering due to L.D. applied directly after vernalization, while late application (L3) causes on the contrary a delay in budding, though the increase in leaf numbers over the S.D. controls is not significant.

Application of L.D. before vernalization appears to have caused decreases in leaf number and time to flowering, as would be expected if the 'parentage' effect can be explained on this basis—but the reductions just fail to reach the conventional 5 per cent. significance level. It would seem probable, however, that this explanation is correct, but perhaps a somewhat longer exposure than 4 weeks would be required to secure an effect as great as that associated with L.D. parentage.

TABLE V

Effect of time of application of 4 weeks' L.D. Variety 'Indian Summer'. Experiment started 16.1.51; 6 replicates

	S.D.	L1	L2	L3	L4	Sig. diff.
Leaf-number increment .	27·3	25·5	22·7	28·3	27·5	1·98
Days to budding after end of vernalization . .	59·0	56·7	50·5	66·0	58·8	4·32

DISCUSSION

The experiments described above have established that a limited period of L.D. treatment will accelerate the process of flower initiation in the Chrysanthemum and reduce the number of leaves produced. These results therefore fully confirm Chouard's observation. In view of the fact that the Chrysanthemum has always been regarded as a strict S.D. plant it is interesting that the so-called unfavourable day-length can have a 'favourable' effect. The effect of L.D. depends, however, entirely on its duration and the time of application; also, the state of vernalization of the plant appears to be important, the effect being maximal with full vernalization. In the variety used here, 'Indian

Summer', a period of 4 to 6 weeks immediately after vernalization appears to be optimal. Application before vernalization seems to be effective also and is probably identical with the 'parentage effect' noted previously.

Though this L.D. effect would appear to be somewhat analogous with S.D. induction of unvernalized winter rye (Purvis and Gregory, 1937), there are important differences: for whereas in rye S.D. treatment is effective only with unvernalized plants, in the Chrysanthemum L.D. treatment is most effective with completely vernalized plants, and fails altogether unless some exposure to low temperature is given.

Nothing is known about the mode of action of L.D.; it seems, however, that L.D. treatment somehow 'reinforces' the action of vernalization when further cold treatment itself would have no additional effect. As has been suggested before (1950), vernalization is likely to have a pronounced effect on the auxin metabolism of the plant and, judging by internode elongation, &c., leads either to increased auxin production or to its greater effectiveness in the plant. L.D. also increases the length of internodes, again presumably by affecting auxin relations. The fact that (1) prolonged L.D. treatment cannot substitute for vernalization, and (2) the effect is maximal with complete vernalization, suggests strongly, however, that the two effects are operative at different stages in the train of reactions leading to flower initiation.

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A Note on the Submicroscopic Structure of Pectin in Collenchyma Cell Walls

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In a recent article in this journal, Roelofsen and Kreger (1951) have made a number of pronouncements concerning the condition of the pectic substances in the walls of collenchyma cells in *Petasites vulgaris*. They were led to choose this particular cell type in view of the high pectin content demonstrated some years ago in a publication from this laboratory (Preston and Duckworth, 1946), and our experience both of this particular material and in the study of wall polysaccharides generally leads us to believe that some of their conclusions should not be allowed to pass without comment. In particular it is felt that the evidence fails to demonstrate any *high degree* of axial orientation of submicroscopic pectin fibrils.

The results which Roelofsen and Kreger present are derived from two sources: (*a*) optical, X-ray, and electron microscopic examination of 'pectin skeletons' obtained by the removal of cellulose from bundles of collenchyma cells, and (*b*) two X-ray diagrams of a bundle of collenchyma cells, one taken before and one after pectin extraction. These may well be taken in turn.

(*a*) *Pectin skeletons.* In assessing the value of the optical observations on the pectin skeletons it is important to consider the probability that a quantitative extraction of cellulose has been achieved. It is therefore a little unfortunate that the extraction was performed by immersion of collenchyma bundles for 1–2 months in a solution of cuprammonium made by adding concentrated ammonia to an excess of cupric hydroxide powder. In the first place, solutions made in this way (instead of by the standard method in which powdered copper is dissolved in ammonia under controlled conditions) are seldom adequate solvents for cellulose. In the second, no mention is made of any attempts to ensure a low nitrous acid content, and the solution is apparently performed in air instead of under nitrogen. Under these conditions it seems inevitable that oxidation will occur and the cellulose be partially reprecipitated as cellulose II. Disappearance of anistropy is hardly satisfactory evidence for extraction of cellulose, and it is difficult to ensure the absence of this polysaccharide in the bulk of the material by staining reactions in chlor-zinc-iodide on small parts of it. Proof that the extraction of cellulose is quantitative rests entirely, therefore, upon the X-ray diagrams.

Of these, two are presented. One of them (Pl. 1, Fig. 1 of Roelofsen and Kreger) is a powder diagram in which, as Roelofsen and Kreger themselves point out, it is impossible to decide unequivocally whether the diagram is derived from cellulose II or from pectinic acid. The second diagram, of

stretched pectin skeletons, is more interesting. This consists in the main of two rings, an outer one corresponding to an interplanar spacing of 4·18 Å (and therefore to the meridional (103) reflection in pectin fibres; Palmer *et al.*, 1947) and an inner one corresponding to 6·5–7·1 Å (analogous to the equatorial 6·76 Å equatorial arc in pectin fibres (Palmer *et al.*, *loc. cit.*)). The outer ring is accentuated along the meridian; the inner one is said to be accentuated along the equator (this is not evident on the published diagram, but no doubt the lack of clarity is due to loss on reproduction). The diagram does, therefore, in the absence of any other known chemical substance in large quantity, provide presumptive evidence of the presence of a crystalline pectic compound. It cannot, however, by its very nature demonstrate the absence of cellulose. Further, the appearance of the inner ring in the published diagram provides little evidence of axial orientation; certainly the outer ring indicates a tendency towards such an orientation but, and particularly if cellulose or some other contaminating substance *is* absent, there must occur very substantial angular dispersion. To refer to this as axial orientation without qualification would seem therefore to present a somewhat exaggerated picture.

The same conclusions may be drawn from the optical evidence. The form-double-refraction curve which can be drawn from the data presented is exceedingly irregular. This is possibly due to the unfortunate choice of a method of imbibition, using many liquids instead of mixtures of only two, which has long been known to be unsatisfactory. It does, however, demonstrate a minimum (and therefore intrinsic) path difference (p) of -6×10^{-7} cm. Now the birefringence ($n_\gamma - n_\alpha$) of well-oriented pectic acid is about 6×10^{-3} (Wuhrmann and Pilnik, 1945). Hence, since

$$p = (n_\gamma - n_\alpha)d,$$

the effective thickness, d, of the anisotropic material is about 1μ.

Now a bundle of collenchyma cells in *Petasites* is usually about 12 cells deep and each individual thickening bar is about $2{\cdot}2\mu$ thick in alcohol-dried material. The total thickness of the specimen is therefore about $24\times2{\cdot}2 = 52{\cdot}8\mu$. The cellulose content is of the order of 50 per cent., so that, since this has been removed, the total effective thickness may be about 26μ. Even, therefore, if the beam of polarized light happens to pass by chance through only half of the thickening bars, the discrepancy between the real thickness (*c.* 13μ) and the thickness (1μ) determined from the birefringence on the assumption of good axial orientation is far too large to be ignored. This is equally, though less markedly, true if the substance assumed present is fully methylated pectin instead of pectic acid. The optical observations again, therefore, provide strong evidence for marked disorientation.

This is equally in harmony with the electron micrographs, where evidence for orientation is completely lacking.

The general conclusions to be drawn from the observations on pectin skeletons would therefore seem to be that the presence of a *high degree* of axial

orientation is not proved. This lack of orientation could be due, as Roelofsen and Kreger point out, to a shrinkage of the specimens during cellulose extraction. Nevertheless, the marked disorientation observed is at least no guarantee of a high degree of orientation before treatment.

(*b*) *The native cell wall.* It remains to examine the evidence concerning the cellulose-pectin complex in the native cell wall. It is very satisfactory that Roelofsen and Kreger have verified on this material that the cellulose component is crystalline before drying, just as had already been shown for the cellulose of *Cladophora* and of conifer cambium (Preston, Wardrop, and Nicolai, 1948). The ancillary evidence concerning the orientation of pectic compounds is hardly so convincing. In the first place, observation of a single arc corresponding to a spacing present in many substances besides pectin, including native cellulose, can hardly be accepted as demonstrating even the presence of crystalline pectic compounds. The evidence for a *high degree* of axial orientation also depends entirely on the presence of this so-called arc in a meridional position in untreated collenchyma and its absence in pectin-free bundles. Two points should be made about these two diagrams. Firstly, the arc referred to occurs in the locality of the 021 arcs of cellulose which in the diagram of untreated bundles are not resolved; in addition, the lateral 3·9 Å arcs, which are spread into a complete circle, overlap in this position. The consequent danger implicit in the interpretation of arcs occurring in this position has already been pointed out (Preston, 1946). Secondly, the pectin-free bundle only had been *slightly stretched.* This normally has the effect of separating the 021 arcs, clearing up the meridian, before any visual change occurs in the equatorial arcs. The absence of a meridional arc attributable to pectin might therefore be due merely to stretching.

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Editor's Note. By an oversight Dr. Preston, a member of the Editorial Committee, did not see the paper by Roelofsen and Kreger (*J. Exp. Bot.* **2**, 332) before publication.